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BULLETIN NO. 30

LABORATORY STUDIES OF SLUDGE DIGESTION

BY

A. M. BUSWELL AND S. L. NEAVE

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LETTER OF TRANSMITTAL

STATE OF ILLINOIS

DEPARTMENT OF REGISTRATION AND EDUCATION
STATE WATER SURVEY DIVISION

URBANA, ILLINOIS, June 21, 1930.

M. F. Walsh, Chairman, and Members of the Board of Natural Resources and Conservation Advisers:

GENTLEMEN: Herewith I submit a report of laboratory studies of sludge digestion. This work has extended over a period of more than five years and has resulted in the development of an accelerated process for the stabilization of waste matter. This process was described in a previous bulletin (No. 28).

Respectfully submitted,

A. M. BUSWELL, *Chief.*

I

INTRODUCTION

The sewage-treatment method most widely used by communities of medium or large size involves the sedimentation and separate treatment of the suspended solid materials. The sludges, or settled solids, are fed to an anaerobic bacterial flora which converts them into inoffensive products, the process being termed digestion. The capacity of a plant, treating sewage by sedimentation, is conditioned by the rate at which this bacterial degradation can be accomplished. Combustible gases are evolved during digestion, and these have commercial value when sufficiently high rates of digestion can be maintained.

The anaerobic degradation process is a highly complex series of reactions, but little understood at present. To quote from Stoddart, "useful as the preliminary partition of sewage constituents may be, the final disposal of the putrescible matters is entirely the work of living organisms, and, in the main, of bacteria." Also, "a true understanding of the complicated phenomena involved can only be built up step by step from the results of artificial experiments upon simpler materials, in which the varying factors can be controlled, and which can be repeated at will by independent observers."

The present studies deal with some of the chemical changes occurring during sewage-sludge digestion. The initial plan was to investigate sludge mixtures during the act of fermentation and to formulate a mechanism for the digestion process from observed changes in the more important sludge components. As the work advanced, it became necessary to limit the study more and more to certain substances on account of the diversity of simultaneous biological processes, the mutual interaction of these processes under only slightly varied conditions and the lack of analytical methods for following some of the steps in the digestion.

The study has extended over a period of three years. At its inception, the current views among sanitary chemists held that the decomposition of proteins was the most important step in the stabilizing of sewage solids. Accordingly, the degradation phase of the nitrogen cycle was first given attention. Then commercial emphasis on power-gas production by sludge digestion led to the study of carbonaceous constituents, emphasis becoming centered on grease degradation which appeared quantitatively to dominate gas production. This phase of the study in turn led to an investigation of methane production from lower fatty acids, and to the formulation of a mechanism for anaerobic oxidations which probably has wide biological applicability.

In accordance with the practice in this Division, a close correlation has been maintained between the laboratory investigations and our experimental plant in order that the laboratory findings might be evaluated in terms of practical benefits to the problem of sewage treatment.

The nitrogen studies have shown that the normal digestion process involves liquefying and hydrolyzing the insoluble proteins to soluble split products, some of which are resynthesized into living cells. In acid sludges, these transformations are arrested and the production of offensive alkylamines is favored; degradation of the cellulosic material stops and the sludges retain their hydrophilic nature. But the digestion of grease to lower fatty acids is not arrested and this process aggravates the acid condition unless mixing by circulation or other form of neutralization is resorted to.

Normally the fatty acids produced in the degradation of grease, proteins or cellulosic materials are broken down into methane and carbon dioxide by a group of microorganisms which tend to accumulate in the ripe sludge. Methane production has been shown to be the result of a biological oxidation process involving the addition of water and is of the same general type for all fatty acids. The chemical union of water makes the weight of gaseous end-products exceed the weight of organic acid digested, and when the identity of the latter is known, the products can be calculated; thus a stearic acid soap will produce 150 per cent of its weight of gas, including the dissolved carbon dioxide. The nature and composition of the evolved gases are, of course, dependent upon operating conditions and the buffer capacity of the individual sewage.

Since the composition of a sewage depends not only upon the kind of water supply producing it, but also upon industrial wastes contributed to it, each treatment plant has its own problems. Many of these could be solved by the application of facts widely scattered throughout the field of biochemistry. If the present studies seem at times to wander into realms of academic interest, the diversion is primarily to call attention to the related biochemical findings so that they may be applied where local problems require them.

Acknowledgement is made to the Chemical Foundation, Incorporated, for financial assistance during the latter half of this investigation.

II

HISTORICAL

Controlled digestion of sewage solids dates from the development of the septic tank about 1896 by Cameron of Exeter, England,[11] though before this date unscientifically operated cesspools had been in use.[127] The septic tank is essentially a covered chamber through which sewage flows at a sufficiently reduced velocity to permit sedimentation of suspended solids. The solids then undergo biochemical degradation with the production of an inoffensive humus-like product, which is removed from the tank at suitable intervals. This device reached its peak of popularity in America about 1906, but the need was becoming increasingly apparent for separating the sedimentation and digestion processes, so that gases arising from the latter would not hinder settling. Experiments on double-deck tanks had been instituted by the Massachusetts State Board of Health[40] in 1899, and based on these early trials the Travis or Hampton tank was constructed at Hampton, England, and

patented by Dr. Travis in 1903. Tests made on a Travis tank in the Emscher district (Germany) in 1906, led Dr. Imhoff in the following year to the construction at Recklinghausen of the well-known Emscher or Imhoff tank. An upper section of this tank is designed for sedimentation; the solids fall from it through a slot into a lower section for digestion. Various designs of the Imhoff tank have played a prominent part in America as well as on the Continent. However, the increased digestion capacity attainable by warming the digestion tank, and the high construction cost of the deep Imhoff type, have led in recent years to various tank designs which even more completely separate the sedimentation and digestion process; some of these involve entirely separate units, or a series of remote sedimentation units feeding a common digestion unit.

Whatever the tank design may be, the feature of interest in the present discussion is the digestion of the solids. This process has been defined[68] as "the anaerobic decomposition of sewage [sludge] whereby intensive growths of bacteria directly, or more probably indirectly through enzymes, bring about the liquefaction or gasification of solid organic matters." The result is the rotting of the organic solids to an inert, inoffensive, humus-like residue.

The general types of biochemical reactions operative in this degradation have been summarized by Buswell,[27] who states: "The net effect of all these biochemical reactions is to break down some of the less complex compounds to the gaseous end-products carbon dioxide and methane and at the same time to remove the solution link groups from the more complex compounds, thus leaving a relatively inert solid residue which has a greatly decreased affinity for water." Elsewhere also in his monograph, this author stresses the important changes in physical (colloidal) properties of the solids as a result of digestion; they are probably commensurate in importance with the purely chemical changes but have been neglected in the sanitary field due to the scarcity of workers trained in colloid chemistry. Certainly a jelly, however nonputrescible, is a less desirable end-product than a granular loam; yet the colloidal state represented by the jelly can almost be attained by appropriate mismanagement.

Of the chemical processes occurring in the digestion tank, protein degradation has long been considered the most important. This fact is attributable to the known offensive nature of the products of putrefaction, notably hydrogen sulfide, volatile amines and mercaptans. However, the growing economic interest in the collection of valuable fuel gases from digesting sludge has led to the recognition of other important constituents in sewage solids. The aim at present is a high gas yield and as rapid a digestion as is consistent with the production of an inoffensive product. The control of hydrogen-ion concentration and of temperature has been resorted to, as reviewed by Buswell.[27] The anaerobic tank is becoming less a playground for adventitious microorganisms and more a selected group of workers from whom a certain daily output is expected. The change in viewpoint may be illustrated by the following example.

Imhoff tank operation has always been hazardous in that occasionally, for unknown reasons, violent biochemical action would ensue and cause sludge to foam up out of the tank. The objection to this foaming is the odor and unsightly mess resulting, but the amount of biochemical degradation effected per unit of time during foaming greatly exceeds that effected in a quietly operating tank. Recently a means has been found[28] for maintaining a digestion tank in a condition of controlled insipient foaming, obtaining thereby high rates of degradation and gas production. Thus the much dreaded foaming may become an efficient aid in sewage treatment.

A few investigations have been made to determine the origin of the methane to which the sewage gases owe their value. Bach and Sierp[9] added a number of possible sewage components to digested sludge (which had almost stopped gassing) and observed the volume and composition of the gases resulting from this addition. Their results are summarized in Table I.

TABLE I

BACH AND SIERP'S RESULTS ON GAS PRODUCTION

Material	Per cent digested	cc. gas per gram dry weight	Nature of the gases
Raw meat	91	54.5	CO_2 and N_2; some H_2
Cooked meat	91	46.6	CO_2 and N_2; some H_2
Indigestible meat residue	80	50.	CO_2 and CH_4
Cooked egg white	97	40.	CO_2 and CH_4
Dextrose	100	17.	H_2, CO_2 and N_2
Carrots, fresh	83	526.	CO_2 and CH_4
Carrots dry (100°)	75	528.	CO_2 and CH_4
Raw potatoes	57	247.	CO_2 and H_2
Cooked potatoes	83	410.	CO_2 and H_2
Feces	49	739.	High in CH_4

For comparison it may be noted that sewage sludge digests to the extent of 30 to 50 per cent and yields about 420 cc. of gas per gram of organic matter;[69] the gas contains 60 to 85 per cent of methane, the balance being mainly carbon dioxide.

Favre,[63] from studies on septic tanks concluded that the albuminous material and cellulose digested most rapidly, and that fats were broken down only at the tank surface. O'Shaughnessy[143] studied the chemical nature of fresh and digested sludge, and distinguished two periods in the digestion process; a rapid reduction of 30 per cent in the organic matter requiring 7 to 8 weeks, and a slower change in the physical (colloidal) character of the solids giving a "ripe" sludge in an additional month's time. He observed that even digestion for several years left about 40 per cent of organic matter in the product.

Thumm and Reichle[198] recognized two phases in the digestion process; an initial phase, acid in character, and a later alkaline one. Imhoff's early patents[100] designated these as putrid and odorless phases, though later[98] he adopted the expressions "acid digestion" and "methane

digestion" to describe the two types of reactions. These last two types are still recognized, and the successful operation of a digestion unit seems to depend upon the maintenance of a correct balance between them.

Rudolfs and his coworkers[166] have published extensively on sludge digestion, with emphasis on the gross changes in organic matter, pH control and the quantity of old sludge required in a tank to properly seed the incoming fresh solids.[167] The operating procedures formulated by this group of workers apply to soft-water regions where the buffer effect of high alkalinity is lacking. These, and similar studies,[61] have shown "that apart from a multitude of as yet undetermined influences there are two factors that affect greatly the time required to carry the process to completion. These factors are temperature and reaction. There is a range of favorable temperature and a range of favorable reaction." A temperature between 25° and 28° and a reaction varying between pH values of 6.8 and 7.6 for different plants have been found most favorable for gas production.[61] The operating conditions and gas production in treatment plants here and abroad have been ably summarized by Zack and Edwards.[218] Scant attention has been given to changes in the individual chemical substances in digesting sewage sludge. The sludge seems to have been regarded as a mixture entirely too complex for profitable chemical analysis. Such has not been the case in soil chemistry, so it seems probable that a closer study of sludge chemistry would permit the profitable application to sludge digestion of some of the vast amount of biochemical knowledge which has accumulated in recent years.

III

BIOCHEMICAL CHANGES IN NITROGENOUS MATTER

Nitrogenous Constituents of Sewage

Domestic sewage contains two classes of nitrogenous matter; primary constituents discharged into the sewer as such from domestic and civic activity, and secondary constituents derived from the above by chemical or biochemical processes in the sewer during transit to the treatment plant, in the soil and entering the sewer by infiltration or on the streets and being contributed in the runoff. Some of these constituents are small in quantity and sporadic in occurrence, such as picoline carboxylic acid from the soil in the rainy season, and can be omitted. Others like the preformed humic acids of sewage, are best discussed with their carbonaceous precursors in a later section. The quantitatively important and relatively persistent nitrogenous substances of human origin are shown in Table II. These materials are partly in solution and partly contained in the settleable solids; since the anaerobic treatment of sewage is concerned principally with the latter, they have received most attention.

TABLE II

NITROGENOUS WASTE PRODUCTS OF HUMAN ORIGIN

		Grams per capita per day
Urine:	Urea	35.
	Creatinine	1.0
	Uric acid	0.75
	Hippuric acid	0.7
	Ammonia	0.65
	Thiocyanic acid	0.15
	Indican	0.01
Feces:	Bacterial protein (dry)	6. to 7.
	Indol and Scatol Histamine Undigested protein	4. to 5. (as N)
	Guanine and adenine	0.1 to 0.2
	Xanthine and Hypoxanthine	traces
Miscellaneous:	Casein Nucleoprotein Albumin Globulin etc.	(no estimate)
Secondary products:	Ammonia	
	Free Amino acids	
	Carbamino compounds	
	Aliphatic amines	
	Pyrimidine and piperazine nuclei etc.	

The soluble nitrogenous constituents in raw sewage have already been discussed by Neave and Buswell[13c] in regard to the sanitary significance of their routine determination. In brief, the findings showed a wide variation in sewage composition with the time of day.

	Nitrogen as percentage of total present			
	8:30 a. m.	10:00 a. m.	1:30 p. m.	
Urea	63.5	40.7	35.6	
Ammonia	23.5	45.5	50.3	
Amines	5.6	3.2	2.6	
Protein, etc.	7.4	10.6	11.5	
Total N present	37.9	44.2	40.1	mg. per liter

To these values, we may now add:

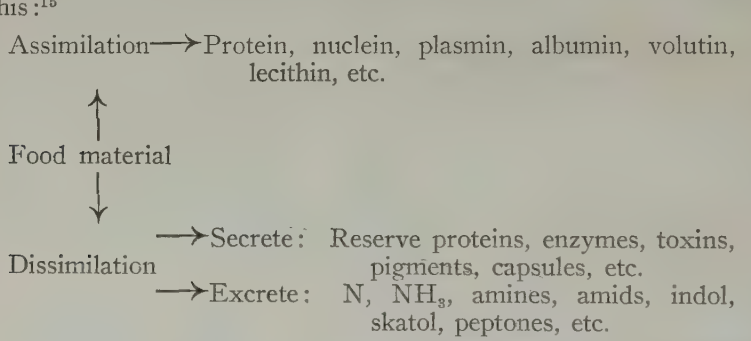
	As nitrogen, mg. per liter
Total amino acids	0.8 to 10. (depending on age of sewage)
Cystine	1.0 to 3.0
Cysteine	(none in fresh sewage)
Histidine	present
Arginine	trace
Lysine	(not found)
Xanthine and Hypoxanthine	trace
Cytosine	(not found)
Creatine and creatinine	trace
Uric acid	trace

Of the above amino nitrogen, 3 to 10 per cent is associated with calcium carbonate as carbamino compounds in this hard-water region.

The insoluble nitrogen associated with the settleable solids includes bacterial bodies (nucleoproteins), various combined and coagulated proteins, animal and vegetable fibers, etc. Quantitatively it varies between wide limits; an approximate value for this community is 0.05 to 0.06 pound of dry solids per capita daily,[34] these solids containing 2.5 to 3.5 per cent nitrogen. Since most of the insoluble protein is combined or denatured, its character is revealed best by the distribution of nitrogen after hydrolysis, as will be shown below.

Bacterial Degradation

The bacterial breakdown of the nitrogenous substances is complicated, and the products are so dependent upon environmental conditions (often local or transitory) that generalized statements are hazardous. Anaerobically the hydrolytic and reduction reactions predominate, sometimes merely as a source of energy, and sometimes as steps in anabolism. The obligate anaerobes commonly derive their energy from carbon transformations, and proteolysis and kindred reactions are ancillary anabolic phases; however, if carbohydrate or other suitable material is lacking, the catabolic phase also is dependent upon proteolysis. The predominant reactions are biochemical and fit into such a scheme as this:[15]


Assimilation
Protein, nuclein, plasmin, albumin, volutin, lecithin, etc.
Food material
Dissimilation
Secrete: Reserve proteins, enzymes, toxins, pigments, capsules, etc.
Excrete: N, NH3, amines, amids, indol, skatol, peptones, etc.

Insofar as information is available, the degradation of the individual nitrogenous compounds will next be considered.

Proteins. Pure proteins are readily attacked when other forms of nitrogen also are present[20],[185], and proteolytic bacteria capable of digesting coagulated proteins have been found in concentrations up to a million per cubic centimeter in sludge digestion tanks;[96] a number of the proteolytic clostridia have been identified,[74] particularly the fecal group including *Cl. welchii* and related forms which are known to take proteins down to amino acids.[71],[76],[214],[215] *Bacterium coli* is said to take casein down only to peptone,[192] though macerated dead *coli* has shown ability to split glycyl-dl-tyrosine.[129] The present evidence is that the dipeptid anhydrides (dioxypiperazine derivatives) are not readily split by enzymes,[107] and even with a mixed soil flora unattacked protein residues are left by the organisms;[162] a similar indigestible residue

seems to persist in anaerobic sludge digestion and to contribute largely to the so-called "humus" produced by this process.

Amino acid production is increased by carbohydrates,[49] but too high a concentration of some of the amino acids can definitely retard bacterial growth.[73,217] At least some of the putrefactive anaerobes reduce the resultant amino acids farther to ammonium salts of the corresponding fatty acids.[145]

Amino acids. In addition to deamination by reduction, the amino acids may be decarboxylated to amines, especially in acid media; thus, leucine produces isoamylamine[3]; valine, isobutylamine; lysine, cadavarine, aspartic acid, ethylamine; glutamic acid, propylamine; phenylalanine, beta-phenylethylamine; tyrosine, para-hydroxy-phenylethylamine; histidine, beta-imidazolethylamine (histamine); arginine gives urea plus ornithine or putrescine[101,102] or may go directly to agmatine and this subsequently give putrescine;[160] tryptophan readily gives indolethylamine, though the tyrosine and tryptophan rings may even be ruptured with complete degradation to lower fatty acids and ammonium carbonate.[189] Amine production from glycocoll, alanine, norleucine and isoleucine is probable but not proved; decarboxylation of serine, cysteine and proline has never been obtained, though proline may undergo a reductive opening of the ring to delta-amino-valeric acid; in this connection it is interesting to note that arginine never gives the corresponding guanidino-valeric acid.

These decarboxylations all require an acid medium, and much of the odor from an acid, poorly digesting sludge is due to the high ratio of primary amine to ammonia nitrogen; we find this ratio to be 1:4 to 1:10 or more during normal sludge digestion, but as high as 1:1.5 in a digesting mixture at pH 5.5. A pH of 5.0 to 5.2 is reported to favor histamine and tyramine production by fecal organisms[84]; in fact "the production of amines from amino acids by microorganisms seems to be a protective mechanism and is resorted to when the accumulation of hydrogen-ions within the organism's protoplasm is incompatible with its normal life processes."[84] It may be noted in passing that the reverse reaction, amine utilization, is a property of some aerobic bacteria,[50] but this ability has not been found in anaerobic forms.

Aspartic acid is able to give succinic acid under both aerobic[22] and anaerobic[45] conditions; glutamic acid probably suffers a similar deamination, though this has not been demonstrated.

Histidine has been found to give not only histamine, together with some acetic and butyric acids and ammonia,[21,84,88] but also urocanic acid (beta-imidazolacrylic) by members of the colon-typhoid group.[157] Tyrosine, likewise, may give tyramine,[84,171,172] or, with *B. coli communis* in the absence of lactose, p-hydroxy-phenyllactic acid,[172,173] or p-hydroxy-benzaldehyde and p-hydroxy-benzoic acid, the former being regarded as an intermediate in oxidative melanin (humin) formation;[91] or, lastly, it may give phenol[84,181] in alkaline media. The phenol content of digesting sludge is only a few milligrams per liter, but even this small amount probably aids the growth of obligate anaerobes, as will be mentioned again later.

The degradation of tryptophan is commonly to indolacetic acid by many organisms,[137] or less commonly to indol.[16]

In addition to these degradation reactions, free amino acids may disappear from the medium by bacterial anabolism, a condition observed in the late stages of sludge digestion (see below). On the other hand, they may be synthesized from ammonia and degradation products of sugars and fats.[15],[110]

Urea. The bulk of the urea in sewage is of primary excremental origin, though secondary contributions can result from proteolysis (especially from the hydrolysis of arginine) and from nucleic acid degradation (from the purines and pyrimidines).

Microorganisms capable of hydrolyzing urea to ammonium carbonate are prevalent in polluted water[170] and soil,[188] and in manure[70] are often associated with hippuric acid strains.[186] Old cultures of these forms tend to lose their power of urease production, but this can be revived by sodium citrate treatment;[164] also, in the case of the *coli* group, leucine is said to be essential to the production of this enzyme.[104]

Purines and Pyrimidines. Uric acid, guanine, adenine, xanthine and hypoxanthine are primary excremental products[175] together with undecomposed nucleoproteins. They may appear also as secondary products of nucleic acid destruction or of purine synthesis by bacteria.[124]

Uric acid is readily destroyed by intestinal organisms with the production of ammonia, carbon dioxide, allantoin, urea and oxalic acid as end-products. With a sodium phosphate-acetate medium, containing 200 mg. per liter of uric acid and inoculated with an infusion of feces, Morris and Ecker[132] found 6 per cent destroyed in one day, 84 per cent in 3 days and complete destruction in 5 days; with an organism from chicken excreta, destruction was complete in only 36 hours.

Regarding the other purines, guanine has been shown to break down into xanthine and ammonia, or into guanidine, urea and ammonia, and these products, together with hypoxanthine, can be split farther by intestinal bacteria with the liberation of ammonia as the nitrogenous end-product.[15] The combined purines of meat have been shown to suffer almost complete destruction during spoilage, especially by *B. coli communis.*[62] The oxidation of adenine to hypoxanthine, and of this together with xanthine, to uric acid can be accomplished anaeorbically by tissue and milk enzymes in the presence of hydrogen acceptors.[131]

The pyrimidines have been less completely studied. Cytosine can be transformed almost quantitatively into uracil by putrefying tissues[103] and by *B. coli.*[82] The phosphopentosides, adenosine and guanosine, can yield nearly theoretical quantities of ammonia with intestinal organisms.[194]

Miscellaneous. Hippuric acid is readily converted into benzoic acid and glycocoll by many soil and water forms.[15]

Carnosine is completely utilized by *B. pyocyaneus* with the production of acetic and butyric acids, ammonia, etc.[88]

Creatinine is converted by putrefactive bacteria into beta-methylhydantoin,[1] the subsequent fate of which is unknown. In view of the

ready hydration of creatinine to creatine in alkaline solution,[179] the latter form probably occurs in sewage, and it may break down by hydrolysis into ammonium carbonate and methylamine; this mechanism has not been substantiated.

Experimental

The soluble nitrogenous components have received little attention in the present study because they occur in the digestion chamber or tank only in the liquor associated with the entering settleable solids, and very early in the digestion period they are quantitatively overshadowed by soluble nitrogenous compounds derived from the fermenting sludge.

Apart from ammonia, urea is the predominant, soluble, primary component. Its decomposition has been followed in a sample of settled sewage stored at 26-27°, by determining both the liberated ammonia and the residual urea by the Marshall urease method as modified by Van Slyke and Cullen.[205] The following results were obtained:

Time of storage hours	Residual Urea-N mg. per liter	Per cent urea destroyed	Valves of k for $kt=\ln(a/a-x)$
Initial	18.83	0.0	.....
4	18.11	3.8	0.0098
23	11.60	38.4	.0209
46	6.27	66.7	.0240
69	3.09	85.3	.0261
92	1.24	93.4	.0295
115	0.2	98.9	.0394

Since the ammonia-N increased in proportion to the urea disappearing, the destruction of the latter was a simple hydrolysis; hence the acceleration in velocity, shown by the above "constants" for a first-order reaction, indicate a progressive establishment during storage of more favorable hydrolytic conditions, and suggest that urea destruction in a normally operating anaerobic tank might be many times as rapid as here indicated. Confirmatory evidence for this view was obtained by adding 10 to 20 mg. amounts of urea to liquor drawn from a sludge-digestion tank; destruction was complete in 24 to 48 hours at room temperature.

The settleable solids of our sewage contain nitrogen to the extent of 2.5 to 3.5 per cent of their dry weight; if this nitrogen is regarded as associated wholly with proteins, the sewage solids have the following approximate organic composition in per cent of their dry weight:

	Undigested Solids *per cent*	Digested Solids *per cent*
Protein (6.25xN)	19.4	12.5
Humic acids (pyridine soluble)	4.0	8.6
Crude fiber	10.8	9.8
Grease and soaps	25.2	6.9
Total	59.4	37.8
Volatile matter by analysis (100%-ash)	60.9	39.6

Allowing the total nitrogen present to represent 100 per cent, acid hydrolysis shows the following N-distribution for the proteins:

	Undigested *per cent*	Digested *per cent*
Humin-N	10.57	21.35
Ammonia-N	13.27	12.01
Amino-N	62.21	64.42
Non-amino-N (by difference)	13.95	2.22

Further separation of the 62.21 per cent of amino-N in the fresh solids showed:

	Per cent
Mono-amino-N	45.42
Di-amino-N	14.28
Tyrosine (+ tryptophan)*	2.03
Undetermined	0.48
	62.21

*Tryptophan is almost wholly destroyed during acid hydrolysis, yielding humin (melanin).

Fresh solids are always characterized by a large non-amino-N fraction, which disappears during digestion. This fraction has been analyzed by precipitation of the copper and silver salts[111] of the purines (together with proline, leucine and norleucine) and shown to consist almost entirely of purine bases, presumably derived from nucleoproteins. The presence of phosphates in digested sludge-liquor mixtures (about 170 mg. per liter), and their absence in the water-supply, support this view; in fact, free phosphoric acid might prove to be a useful index of "ripeness" or completeness of digestion.

Further light was thrown on the changes in nitrogen distribution during digestion by comparing several samples of freshly settled solids in Imhoff tanks and separate sludge-digestion units in operation at our experimental plant. Figure 1 presents graphically the averages of the individual values in Table III; appended to the latter, for comparison, are the corresponding results reported for a soil analysis.[152] The most striking result is the disappearance of the non-amino (purine) fraction, with a slight increase in ammonia nitrogen, and presumably an increase in the number of bacteria; the latter would pass out of the tank with the liquor and feed the protozoa and metazoa of the subsequent aerobic steps in sewage treatment. Insofar as nitrogen transformations alone are concerned, the best criterion of completeness in sludge digestion is the decrease of purine N from an initial 25 to 35 per cent of the total for strictly fresh solids to 1 or 2 per cent of the total in thoroughly digested material.

The apparent increase in amino-nitrogen, shown in Table III is due to the liquefaction of part of the solid matter and a resultant concentration of insoluble components in the residue; thus if a 33 per cent

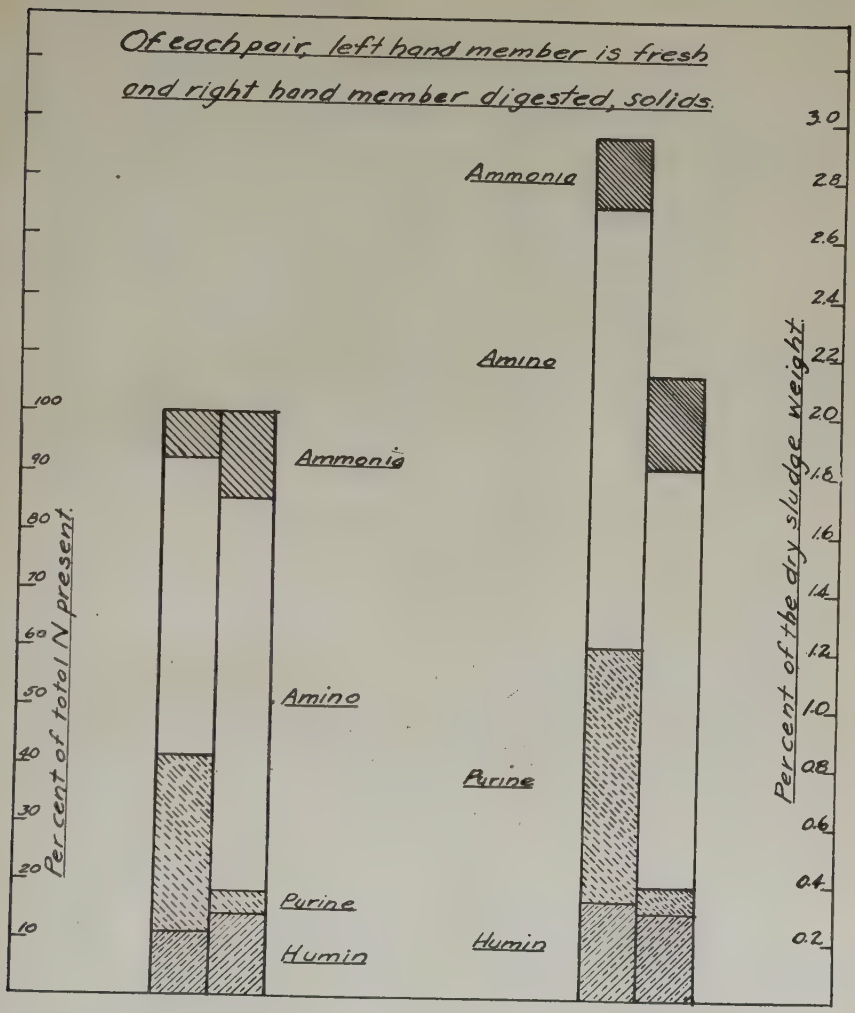


Fig. 1—Ammonia, amino, purine, and humin nitrogen in hydrolyzed sewage protein. Plotted as per cent of total nitrogen present (left) and as per cent of dry sludge weight (right).

reduction in total weight of solids be assumed and the percentages of nitrogen in the digested solids be corrected for this shrinkage, then the following comparative values are obtained, considering only the averages

TABLE III

NITROGEN DISTRIBUTION IN HYDROLYZED SEWAGE PROTEINS

	Sample No.	Total N in dry solids Per cent	Humin N* Per cent	Ammonia N* Per cent	Amino N* Per cent	Non-amino N* Per cent
Fresh solids:	4-1066	2.84	8.7	11.4	54.1	25.8
	4-1072	2.78	20.4	7.6	49.3	22.7
	.....	2.94	10.5	13.3	62.3	13.9
	4-1084	3.27	10.3	7.2	47.0	35.5
	4-1164	3.24	8.5	5.5	58.2	27.8
	4-1174	2.66	10.3	6.9	48.9	33.0
	4-1246	3.21	9.4	8.1	51.1	31.4
	Averages	2.99	11.2	8.5	53.0	27.3
Digested:	7A-1365	2.12	15.8	13.5	59.6	11.1
	7B-1367	2.50	13.6	14.3	67.3	4.8
	.....	2.34	21.3	12.0	64.4	2.3
	7A-1407	1.78	14.7	14.7	69.2	1.4
	7B-1408	2.16	14.5	12.9	72.3	0.3
	10-1409	2.09	11.6	16.9	70.1	1.4
	Averages	2.16	15.2	14.0	67.1	3.7
Soil (Potter & Snyder, 1915):			6-9	18-27	31-39	2-6

*Based on total Kjeldahl N=100 per cent.

from Table III and expressing the nitrogen fractions as grams present in 100 grams of initial dry solids:

	Total N	Humin N	Ammonia N	Amino N	Non-amino N
Fresh	2.99	0.33	0.25	1.58	0.83
Digested	1.44	0.22	0.20	0.97	0.05
Per cent loss.....	51.8	33.3	25.0	38.6	93.9

In order to follow more closely these nitrogen transformations during digestion, two series of one-liter bottles containing sewage solids were allowed to digest at room temperature, one bottle being removed from each series at intervals for analysis. One series was allowed to develop the acidity (pH 5.5 to 5.0) characteristic of the so-called "acid digestion," and the other was maintained at pH 6.9 to 6.4 by additions of lime. Before analysis, the bottle contents were separated on a suction filter into a sludge and a liquor fraction, in order that liquefaction of solid nitrogen might be followed. The data presented in Table IV show the rapid destruction of urea and purines, and the slow liquefaction of solid nitrogen at pH 5.0 compared with that at pH 6.4. It should be emphasized, however, that in the acid type of digestion the production and ready evolution of hydrogen sulfide, the formation of volatile

free fatty acids and the liberation of alkylamines (included here with free ammonia) all contribute toward the offensive character of an "acid" sludge; analytical data on any single metabolite cannot adequately portray this character.

An interesting observation from these digestion experiments is that, during the active period of decomposition, the total of all forms of nitrogen remains nearly constant, and the result of digestion in a closed system is mainly a readjustment of the various nitrogen combinations. While slight deviations in total nitrogen are found among the various bottles, due to difficulties in sampling a heterogeneous mixture like sludge, the average of all final nitrogens shows a loss of only 5.0 per cent of that initially added. More precise determinations of this loss were obtained in later studies on gas-production, and, at the risk of some discontinuity in theme, will be discussed at this point.

The usual presence of nitrogen in the gases evolved from sludge digestion has led to the assumption that the biochemical degradation of organic matter resulted in its production, and a number of suggestions have been offered as to its origin. Groenewege,[77] working with septic tanks, suggested nitrification in the surface scum since Winogradsky and Omelianski[140] had found very low oxygen tensions sufficient for nitrite production; the resulting nitrite was thought to diffuse down into more anaerobic regions and there suffer denitrification; however, Grownewege's use of methylene blue as a criterion of a nitrifying zone, in a scum normally having an acid reaction, is misleading in view of the marked change in oxidation potential of this dye with hydrogen-ion concentration. Deamination reactions, resulting from pre-formed nitrites in the sewage encountering ammonia or amino acids, have been amply discussed by Bach and Sierp;[7] they probably play little part with most sewages. These authors also report laboratory experiments on sewage-sludge digestion, extending over a nine-month period, in which daily samples of the fermenting mixture were obtained by displacement with tap water and shown not to contain nitrates or nitrites; however, Buswell and Neave[30] have pointed out that the gaseous nitrogen collected by Bach and Sierp can be attributed to dissolved nitrogen in the daily additions of water. This sweeping out of dissolved gases had already been observed by Buswell and Strickhouser[35] in treatment-plant operation. Thus the early findings of Popoff,[151] that anaerobic digestion with rigorous exclusion of air showed no evolution of nitrogen, have never been confirmed. The following tests were accordingly made to determine the magnitude of the alleged nitrogen loss.

Various organic materials, in concentrations of one gram per liter of water, were allowed to digest anaerobically under conditions resembling those found in a septic tank or in the bottom muds of lakes and rivers. Complex mixtures, such as the settleable solids in a domestic sewage, were completely broken down in about two months with the production of "humic acids" and the evolution of gas high in methane. Lower carbohydrates and the proteins, or their split products, were rapidly attacked, but grease or soaps and cellulose digested more slowly, while the ligno-celluloses appeared to withstand attack for many months.

TABLE IV

CHANGES IN FORMS OF NITROGEN DURING SLUDGE DIGESTION

(Nitrogen values are mg. per liter for liquors and mg. per gram for dry solids.)

Period of digestion, weeks:		0	1	2	3	4	5	6
		Acid Series						
Liquor:	Free ammonia-N	48.6	56.0	44.8	63.5	82.3	88.0	192.0
	Free amino-N	38.4	209.9	213.6	257.0	200.5	154.1	77.6
	Combined amino-N	80.7	12.5	16.6	16.8	48.7	63.0	103.2
	Urea-N	2.2	0.0	0.0	0.0	.....	.....	.....
	pH	6.9	5.4	5.0	5.0	5.0	5.0	5.0
Dry solids:	Humin-N	1.5	1.4	0.3	0.3	0.7	0.6	0.5
	Ammonia-N	4.7	4.8	5.3	7.0	4.5	4.5	2.5
	Mono-amino-N	23.8	19.4	21.0	19.8	18.1	19.4	20.4
	Di-amino-N	0.7	1.4					
	Purine-N	1.2	0.7	0.0	0.0	.....	.....	.....
Total dry solids, grams		47.9	43.8	40.5	40.4	38.1	38.7	38.4
N in dry solids, per cent		2.90	2.81	2.45	2.42	2.25	2.23	2.23
Total N, all forms, grams		1.321	1.356	1.147	1.277	1.216	1.188	1.344

	Alkaline (Limed) Series			
Liquor:	Free ammonia-N	48.6	43.1	51.1
	Free amino-N	38.4	100.1	122.1
	Combined amino-N	80.7	84.7	129.9
	Urea-N	2.2	0.0	.....
	pH	6.9	6.4	6.4
Dry solids:	Humin-N	1.5	0.6	0.5
	Ammonia-N	4.7	2.6	3.5
	Amino-N	24.5	19.5	10.1
Total dry solids, grams		47.9	32.4	34.0
N in dry solids, per cent		2.90	1.80	1.77
Total N, all forms, grams		1.321	1.205	1.304

All digestion experiments were carried out in carefully stoppered bottles from which evolved gases could be collected over saturated brine for measurement and analysis. When gas evolution had ceased, the bottle contents were analyzed for total nitrogen by Kjeldahl digestion and these values balanced against the nitrogen of the initial bottle contents; any loss should be accounted for as nitrogen in the evolved gases. In most cases this complete balance was not obtained on account of the permeability of rubber connections to atmospheric nitrogen.[55,81,89] Even solid rubber stoppers, over a two month period, permit the entrance of appreciable amounts of nitrogen into the digestion gases, though to a lesser extent when heavily coated with paraffin. Complete confidence can be placed only in all-glass apparatus.

Table V (a) shows the magnitude of the error involved in using rubber connections and stoppers over a four-month period. The initial and final Kjeldahl nitrogen content of the material showed little change, but the gas analyses indicated the evolution of appreciable quantities of nitrogen; these gas values have been corrected by subtracting the

dissolved nitrogen in the initial digestion mixture plus that in the small amount of air remaining in the apparatus at the time of assembling. All bottles were inoculated with nearly "ripe" sewage sludge containing about 2.5 per cent of nitrogen on a dry basis.

In the absence of diffusion of atmospheric nitrogen into the apparatus, the sum of the last two columns should equal the total nitrogen found in the initial bottle contents, whereas in some cases the gaseous nitrogen actually exceeds the total amount present when the bottle was sealed up. Disregarding the gas phase, however, the initial and final Kjeldahl determinations are reasonably consistent and show that no evolution of nitrogen occurred under these conditions, excepting a possible 9 per cent for sodium acetate and 4 per cent for glycocoll.

With paraffin-coated rubber stoppers, but no rubber connections, the results were better as shown in Table V (b). This series was carried out at 37°C. over a two-month period. The gaseous nitrogen

TABLE V

NITROGEN BALANCES ON SUBSTANCES DIGESTED ANAEROBICALLY

(All bottles seeded with nearly "ripe" sewage sludge,)

Substance digested	Evolved gas per gram substance c.c. N.T.P.	Combined nitrogen in bottle Initial grams	Final grams	N_2 in gas grams
(a) Rubber stoppers and connections				
Control	424.1	0.2441	0.2451	0.2034
Sodium formate	469.4	.2360	.2358	.1782
Sodium acetate	538.6	.2558	.2333	.2905
Sodium propionate	1033.1	.2360	.2406	.2930
Sodium butyrate	954.7	.2385	.2400	.3186
Sodium valerate	914.4	.2386	.2400	.2272
Sodium oleate	1302.6	.2327	.2347	.4317
Glycerol	1127.5	.2376	.2303	.2606
Glycocoll	722.8	.4214	.4057	.2229
(b) Paraffin-coated rubber stoppers				
Crude sewage protein	710.4	0.5842	0.5579	0.1865
Same + sewage grease	755.4	.5842	.5692	.1330
Sewage grease alone	716.9	.5558	.5545	.1390
Control	230.0	.5558	.5521	.0841
(c) All-glass apparatus				
Sodium propionate	482.8	0.2954 ±.0059	0.2751 ±.0055	0.0126 ±.002

still greatly exceeds any loss indicated by the Kjeldahl values, and even the differences observed in the total nitrogen are not regarded as conclusive because at this temperature and a pH of 8.0, which was used, some ammonia may have escaped with the evolved gases.

A more reliable balance is shown in Table V (c). This was obtained on an all-glass apparatus in which sodium propionate was fermented at 25°C. over a period of four months. With due allowance

for the probable error of these determinations, the initial and final Kjeldahl values show a loss of between 0.0089 and 0.0317 gram while the gas shows a gain of between 0.0106 and 0.0146 gram of nitrogen. Apparently, therefore, a loss of 3 to 5 per cent of the total nitrogen of the inoculating sludge has occurred under these conditions.

While it is realized that other substances, and other concentrations, should be subjected to this time-consuming method of study, the present results seem to justify the conclusion that under normal conditions of anaerobic decay in nature, in the absence of nitrates, not more than a few per cent of the organic nitrogen is lost as gas over a period of months, and the conventional nitrogen cycles probably overemphasize the destruction of fixed nitrogen during the active degradation of organic matter.

On the other hand, old cultures, in which the concentration of organic food has reached a low level, show a marked evolution of nitrogen when exposed to daylight. This loss is attributed to the development of *B. pyocyaneus,* an organism which utilizes bicarbonate as a source of carbon and derives its energy from the oxidation of ammonia to nitrogen.[188] Even dark brown bottles, transmitting low intensity light of 0.50 to 0.65 micron, permit a heavy green film of these organisms to develop on the illuminated inner wall. The nitrogen production and CO_2 utilization is illustrated by the following data from a culture in a brown (Sodium Hydroxide) bottle which had nearly stopped producing gas and had begun to show *B. pyocyaneus* growth:

		Gas composition	
	Gas production	CO_2	N_2
Age of culture—days	cc. per day	per cent	per cent
24	242.	14.4	3.9
31-(growth visible)	197.	11.9	13.1
47	58.	11.3	15.4
74	3.	7.9	54.9

The remainder of the gas was methane with a trace of hydrogen, oxygen being absent.

Summary of the Nitrogen Data

a. The more important nitrogenous substances in domestic sewage are shown to be urea, ammonia and proteins (including nucleoproteins) and their split products.

b. The degradation reactions for these substances, as reported in the literature, are briefly reviewed.

c. Urea is found experimentally to hydrolyze quickly and completely to ammonium carbonate.

d. Nitrogen distribution in the protein fraction has been ascertained by hydrolysis, and the changes followed in this distribution during digestion. The only significant change is the disappearance of the purine fraction.

e. The digestion process is shown to effect merely a remobilization of nitrogen, during which not over five per cent of that present is lost as gaseous nitrogen.

f. This remobilization is shown to proceed very slowly and incompletely in media at pH 5.0, but rapidly at a pH above 6.4.

g. Possible sources of nitrogen loss are discussed briefly, especially the loss occasioned by the autotropic organism, *B. pyocyaneus*, which is illustrated by experiment.

IV

BIOCHEMICAL CHANGES IN CARBONACEOUS MATTER

Carbonaceous Matter of Sludge

The average analysis for 39 samples of fresh sewage-solids, and a like number of digested ones, showed the approximated organic compositions already mentioned; namely:

	Fresh Solids Per cent	Digested Solids Per cent
Protein (6.25xN)	19.4	12.5
Grease (by petroleum ether)	25.2	8.6
Crude fiber	10.8	9.8
(cellulose	1.43	0.02)
Humic acids (by pyridine)	4.	6.9
	59.4	37.8

The chemical identity of some of the carbonaceous matter in sludge has never been determined. Various substances, characteristic of decomposing plant and animal tissues, are to be expected. Some of these have been found from time to time in settled sludge, using the analytical methods of the soil investigator:[180]

Humic acids—always present.
Hentriacontane ($C_{31}H_{64}$)—present.
Paraffinic acid ($C_{24}H_{48}O_2$)—present.
Lignoceric acid ($C_{24}H_{48}O_2$)—sometimes present.
Alpha-monohydroxystearic acid—not found.
Dihydroxystearic acid—sometimes present.
Resins and esters—present.
Fatty glycerides—present.
Soaps—present.
Agrosterol—not found.
Phytosterol—sometimes present.
Galactans—present (0.6 ppm.)
Pentosans: pentose, 1.6 ppm.
methyl-pentose, 0.6 ppm.
Pentose sugars—not found.

The humic acids referred to in this table were obtained by leaching with 2.5 per cent sodium hydroxide and then adjusting the filtered solution with hydrochloric acid to a pH of 4.8; the precipitate is the so-called beta-humus of Waksman.[208] The figures previously given for fresh and digested sludges represent pyridine-soluble humic acids; when due allowance is made for a 30 to 50 per cent decrease in solids during digestion, these values show no increase in pyridine-soluble humic acids. Humus, determined by successive acid and alkali extractions, is

reported by Bach[6] to vary from 27.4 to 49.0 per cent for fresh sludge and 27.7 to 43.4 per cent for digested material. It seems probable that the so-called humus produced in anaerobic digestion of sewage solids is merely a mixture of pre-formed humus in the influent solids with resistant plant residues and indigestible protein fragments.

If the digestion process involved merely humifying the organic solids, then during the process the sludge composition should undergo a series of changes resembling coal-formation (Table VI).

TABLE VI*

	Volatile matter Per cent	Fixed carbon Per cent
Wood	75 to 79	21 to 25
Peat	70.06	29.94
Lignite	60.67	39.33
Anthracite	6.18	93.82

*Parr, Illinois Geol. Survey, Bull. 3.

Buswell and Neave[21] have shown that such is not the case. A series of sludges showed changes in volatile matter and fixed carbon during digestion which were within the range of variation among the fresh or the digested sludges themselves. This is illustrated in Table VII.

TABLE VII

Volatile Matter

	Sludge as Sampled			Ash-Free Basis			Fixed Carbon		
Material	Max, Per cent	Min. Per cent	Av. Per cent	Max. Per cent	Min. Per cent	Av. Per cent	Max. Per cent	Min. Per cent	Av. Per cent
Fresh solids	70.9	46.5	59.3	97.0	83.9	90.8	9.6	2.2	5.5
Digested sludge	61.2	45.9	54.9	92.7	86.2	89.5	8.8	3.8	6.4

Of the major sludge components, grease showed the greatest decrease in weight during digestion, while, of the nitrogenous matter, only the purine fraction underwent a marked change. Accordingly, grease was suspected of being an important source of methane in the digestion gases, and, in view of the growing economic interest in gas production, an investigation of grease digestion was of more immediate interest than the finer ramifications of the nitrogen cycle.

In order to determine the kinds of substances which give large gas yields when added to well-digested sewage sludge, semi-quantitative observations were made on a number of compounds, as shown in Table VIII. The results are expressed in cubic centimeters of gas (at room temperature) produced by one-gram quantities of the substance in periods up to 3 months. Since digesting sewage-solids will give 400 to 600 cc. of gas per gram of organic matter in about two months, substances giving gas-volumes of this order of magnitude deserve consideration as sources or precursors of gas.

Of the carbohydrates, cellulose, starch and dextrin, the penta- and hexahydric alcohols adonitol, mannitol and dulcitol, and glycerol

are important. The trisaccharide, raffinose, and the glucoside, salicin, produce gas very slowly. The fructosan, inulin, is scarcely attacked. The mono- and disaccharides ferment rapidly, but under the conditions of these tests, usually give hydrogen and CO_2 instead of methane and CO_2 and an abundance of fatty acids.

Proteins and peptones are good sources of gas, but the lower spliit-products and related substances apparently are unimportant.

Of the soaps and fatty acids, those which are not precipitated by the calcium salts of the sludge-liquor, are good sources of gas. The precipitated, or relatively insoluble forms, digest very slowly.

TABLE VIII

GAS PRODUCED FROM VARIOUS SUBSTANCES INOCULATED WITH SEWAGE SLUDGE

(Results in cc. per gram.)

		Gas produced in:			
		2-5 days	2 weeks	2 months	3 months
Carbohydrates, etc.	Cellulose		4.0	400.+	
	Starch	400.+***			
	Dextrin		26.	400.+	
	Raffinose		5.	25.	400.+
	Salicin		4.	19.	400.+
	Adonitol		19.	400.+	
	Inulin		22.5	36.	40.+
	Dulcitol		25.	400.+	
	Mannitol		16.5	400.+	
	Sucrose	400.+**			
	Maltose	400.+***			
	Lactose	400.+***			
	Fructose	400.+***			
	Dextrose	400.+*			
	Glycerol		40.	438.	
	Trimethylene glycol		1.	6.5	15.
Proteins, etc.	Casein		6.	723.	
	Glue (animal)		3.	400.+	
	Beef (Bacto)		4.	562.	
	Blood serum		22.5	400.+	
	Ox bile		22.5	400.+	
	Peptone (Difco)		.....	525.	
	Asparagin		1.5	17.5	200.+
	Sod. aspartate		1.5	7.5	20.
	Glycocoll		1.5	11.5	33.5
	Uric acid		2.5	9.0	18.5
	Methylamine		0.5	25.5	35.0
	Dimethylamine		0.5	25.	
	Sec.-n-propylamine		0.5	1.5	7.5
	Ammon. chloride		.....	32.5	

Soaps, Fats, etc.	Sewage grease (acid)		1.5	30.	400.+
	Sewage grease (alk.)		1.5	400.+	
	Ca soaps (of sewage)		1.0	24.	400.+
	Glycerides (sewage)		1.0	19.	440.
	Soap, Ivory (acid)		1.5	?	
	Soap, Ivory (alk.)		1.5	4.0	
	Milk fat	400.**			
	Olive oil		2.5	400.+	
	Sodium oleate			265.	
	Sodium dihydroxystearate			532.	
	Sodium stearate		2.5	47.0	
	Sodium palmitate			225.	
	Sodium myristate		2.5	75.	400.+
	Sodium laurate			110.	
	Sodium caprylate			115.	
	Sodium heptylate			200.	
	Sodium caproate			135.	
	Sodium valerate			260.	
	Sodium butyrate			825.	
	Sodium propionate			670.	
	Sodium acetate		2.5	340.	
	Sodium formate		2.5	34.5	
	Sodium lactate		40.	440.	
	Sodium oxalate		1.5	11.5	26.5
	Sodium succinate		2.5	10.	27.5
	Sodium malate		1.5	5.5	12.5
	Sodium tartarate		9.	30.5	400.+
	Sodium citrate		24.	400.+	
	Sodium mucate		6.	19.5	33.5
	Lecithin			837.	
	Cholesterol			74.	
Miscellaneous	Control (pH 6.5)		1.5	2.5	7.5
	Control (pH 7.5)		1.5	8.5	20.

* 2 days.
** 3 days.
*** 5 days.

Proteins, carbohydrates and fats all tend to yield high acidities. If the pH falls below 6.4, methane production from the acids is retarded and an abrupt, lethal fall in pH is apt to occur. This acidity may be counteracted in practice in two ways: in soft-water regions, Rudolfs[166] has found it necessary to maintain a high ratio of old sludge (containing both buffers and acid-decomposing organisms) to incoming fresh solids, or even to add lime; in hard-water regions, Buswell[36] has shown mere mixing to suffice. Accumulation of fatty acids, at least up to a concentration of two per cent, is not detrimental if sufficient calcium carbonate is present to neutralize them and if high local concentrations are avoided by occasional mixing.

On account of the quantitative prominence of grease, and its ability to give large gas volumes, the remainder of this study will deal with its degradation and problems arising therefrom.

The Fats and Soaps of Sewage Sludge

The ether-soluble matter of sewage from American cities amounts to about 20 grams per capita per day, as shown by the following values compiled largely from Fuller:[68]

	Grease grams per capita per day	
Lawrence, Mass.	18-20	
Waterbury, Conn.	26	
Gloversville, N. Y.	48	(includes industrial wastes).
Columbus, Ohio	18-27	
Philadelphia	28	
Chicago	23-26	
Urbana, Ill.	15-18	

Commercial attempts have been made to recover sewage grease, particularly by the Kremer tank[68] in Germany, which is said to be profitable only for towns of over 45000 contributors, and the Miles acid process in this country,[128] which effects grease separation by sulfur dioxide and heat. At times of high market, these processes can show a profit. Grease separation by catch-basins, baffles or skimming devices is sometimes practiced to prevent its clogging subsequent units in the treatment process. Much of the grease remains incorporated in the settling solids, and even grease recovered by skimming is usually added to solids entering the digestion tank.

Composition of Grease. The combined extracts from 77 samples of local sewage solids over a 3-month period represented an average grease content of 29.2 per cent for the dry solids. The grease is a dark reddish-brown viscid product with a mild odor suggestive of a lanolin-cocoanut oil mixture. It softens at 64° and melts at 68° to a reddish-brown oil. The acid value is 66.6 milligrams KOH per gram and the iodine number (by Wijs' iodine monochloride) is 55 per cent. At times the grease contains nitrogen up to 0.18 per cent, probably due to lipo-

proteins since the nitrogen is not water-soluble but can be removed by acid hydrolysis. About half of the grease is present as soaps:

	Per cent
Soaps (Na, Ca, Mg, Al, Fe)	49.8
Glycerides	33.8
Mineral oils	3.2
Unsaponifiable solids (resins?)	7.1
Moisture	5.5
	99.4

The soaps, separated by steam-distilling off the glycerdies and mineral oils, showed considerable variation in composition in different samples:

	Sample 1 Per cent		Sample 2 Per cent	
SiO_2	2.14		1.72	
Fe	2.82		0.34	
Al	1.30			
Ca	12.15		7.32	
Mg	0.40		0.06	
Na + K	1.70		0.63	
Fatty acids	79.49	(by difference)	89.83	(by difference)
	100.		100.	

The mean molecular weight of the fatty acids in the soaps was 225 to 250, and of the total fatty acids about 270.

Sewage grease may, therefore, be characterized as a mixture of glycerides and soaps (predominantly lime soap in this hard-water region) of higher fatty acids in which the saturated ones predominate. In confirmation of the findings of O'Shaughnessy[143] and of Bach and Sierp,[9] we have noted a marked increase in unsaturation during digestion, the iodine value increasing from 50 to 60 per cent up to 100 or 150 per cent.

Historical. Little attention has been paid to the behavior of fats during digestion. In fact many treatment plant operators have erroneously assumed no decomposition of grease in the process.[197] At least partly responsible for this idea was the observation that the percentage of ether-extractable material showed no change in the digested product; however, as Buswell and Neave[32] have pointed out, a decomposition of 30 to 50 per cent of the organic solids during digestion would necessitate a corresponding destruction of grease if the percentage is to remain constant. Favre[63] concluded that some degradation occurred in a septic tank, but only at the surface. In a summary of operating data, O'Shaughnessy[143] states: "the analysis of the final product showed that a little over one-third of the fatty material and about one-fourth of the nitrogen were lost." The fatty material in this case represented 22.2 per cent of the dry fresh solids, and had a saponification equivalent of 267.3. In this country Riker[161] has observed that sludges of the

lowest fat content came from Imhoff tanks giving the least operating trouble.

In the literature, some confusion has arisen through a failure to distinguish mere lipolytic splitting of fats into glycerol and free acids from the actual degradation of the fatty acid chains; both are referred to as decomposition of fats. Here we are concerned primarily with carbon-chain degradation.

Lipase is produced by many bacterial species, though its activity is markedly influenced by catons present in the culture.[202] Even some spores contain this enzyme.[168] The splitting off and utilization of glycerol is a common reaction, though aerobically the lipases of *B. pyocyaneus* and *B. prodigiosus* can also synthesize esters from a number of monohydric alcohols and glycerol; the *coli*-group does not produce synthesizing lipases.[203] Lipolytic enzymes have been found in samples from a sewage treatment plant.[96],[196] To the amino acids has been attributed the ability of hydrolyzing esters, but recent careful work indicates no such action beyond that attributable to changes in the reaction of the medium.[24]

Cytological studies have shown definite fat inclusions in bacterial cells,[57] and in fungi;[177] this fat forms at the expense of sugar,[12],[19],[193] alcohol,[119] or related substances,[187] and, in the case of the fungi, is the more saturated the higher the temperature of the culture.[144] Some organisms can utilize fats directly, notably the tubercule baccillus,[176] while soil forms, especially the fungi, even decompose paraffin.[65],[156] Decomposition of mineral oil by soil forms has been reported,[17] but the evidence, being merely a change in specific gravity, is not very conclusive. By growth on gelatine, with and without fat, von Sommaruga[207] determined the titratable acids produced by a long series of saprophytes and pathogens, and fungi; unfortunately acid production from the gelatine obscured the results. In the soil, unquestionable disappearance of added fat was shown[165] and attributed mainly to fungi, though evidence was soon forthcoming that several soil bacteria could not only split triglycerides, but also decompose the fatty acid chains.[115],[174] In a review of the question, Rahn[155] assembled the findings of the earlier work on rancidity of fats, cheese-ripening and soil studies, concluding that aerobic fatty acid degradation can occur in the presence of nitrogenous matter, and that anaerobic decomposition never occurs. In fact the marked resistance to destruction led to the idea that pure fats and soaps had strong germicidal powers;[78] proponents of the idea merely had to cite the preservation of some fish in oil, and the lethal effect of fatty acids on some bacteria.[130] Recent work has shown that commercial soaps and lower fatty acids have little germicidal value[210] over the ordinary range of hydrogenion concentration.[56] In nature, fats are resistant to bacterial attack because they tend to occur in dense aggregates which do not contain the other elements necessary for bacterial nutrition.[219] In sewage sludge the finely divided nature of the grease and its distribution amongst fibrous and nitrogenous matter probably contribute largely to its degradation.

On the other hand, this same distribution of grease contributes to the viscosity of the scum which forms on digestion tanks; it tends to

bind together the fibrous material into a tough surface mat. In discussing liquor circulation as the remedy for scum formation, Buswell[29] points out that this viscosity can be lowered by (a) change in pH, (b) increase in temperature, and (c) dilution of the colloid.

Experimental. Preliminary studies by Buswell and Neave,[32] in which the course of grease digestion was followed in digesting sewage solids, showed that even in acid media a rapid destruction of fats and soaps occurred with the production of lower fatty acids. Some of the lower fatty acids fermented farther to give methane and carbon dioxide. In this acid medium (pH 5.0-5.5) the rate of digestion, as measured by gas production, was roughly proportional to the grease-content of the solids fed; a scum containing 73 per cent grease was the most vigorous gas producer of the samples used. At the hydrogen-ion concentration of these digesting mixtures, proteolysis and cellulose degradation were almost at a standstill, and an opportunity was afforded for studying a predominantly grease-digesting culture. Approximately 75 per cent of the added grease was destroyed in 4 weeks, 50 per cent being recovered as gas and the other 25 per cent as volatile acids (mainly acetic). The data are summarized in Table IX.

TABLE IX

ACID DIGESTION DATA

Time Weeks	Total Solids Grams	Ash Grams	Organic Solids Grams	Grease Grams	pH of Liquor	Lower Fatty Acids in Liquor Grams	Total Solids Liquor Grams	Nitrogen Liquor Grams	Cellulose Grams
0	47.9	12.5	35.4	12.4	6.9	0.2	1.39	0.07	6
1	43.8	11.8	32.0	6.8	5.4	1.4	1.23	0.12	6
2	41.0	11.3	29.7	5.3	5.0	1.9	0.99	0.15	5
3	40.4	11.5	28.9	4.9	5.0	2.8	0.93	0.28	6
4	38.1	10.2	27.9	4.0	5.0	3.8	0.87	0.37	____
4*	38.6	12.5	26.1	3.6	6.0	5.2	0.89	0.41	____
5	38.4	11.1	27.3	5.1	5.0	3.6	0.82	0.33	____
6	37.8	11.0	26.8	4.6	5.0	3.7	0.72	0.61	____
7	36.7	10.6	26.1	4.6	5.0	4.1	0.80	0.37	____
7*	37.4	12.6	24.8	2.7	6.4	3.2	0.56	0.58	5.5
8	35.4	9.9	25.5	3.8	5.0	4.2	0.72	0.47	6.0
9*	39.0	13.7	25.3	2.7	6.4	2.7	0.51	0.70	5.5

*Limed.

Even in these acid cultures, the nature and quantity of volatile acid may be influenced by the digestion of substances other than grease. For a study of the chemical nature of grease degradation, therefore, it was necessary to use cultures free from these complications encountered in sludge digestion.

Attempts to obtain pure cultures of grease-digesters, by inoculating agar containing soaps or higher acids with material from actively digesting sludge solids, were not very successful; only in the presence of

large amounts (1 per cent) of peptone were grease-digesting colonies secured, and only when solid sludge material was used as inoculum did gas result. The best medium for gas production was found to be distilled water, 1 liter; agar, 2 grams; $Na_2HPO_4.12H_2O$, 10 grams; meat extract, 4 grams; NaCl, 2 grams; K_2SO_4, 2 grams, and 10 grams of fatty acid or other nutritive. Deep agar shake-cultures, sealed with vaseline after inoculation, gave the following average counts (per cubic centimeter of inoculum) in four series of cultures (Table X).

TABLE X

BACTERIAL COUNTS IN SYNTHETIC MEDIUM

Nutritive added	Average count in 6 days	Relative gas production
Glucose + amyl alcohol	58	+++
Glucose + butyl alcohol	54	++
Glucose + ethyl alcohol	54	+++
Glucose + Sodium propionate	45	+++
Glucose + Sodium valerate	44	++
Glucose + Sodium butyrate	38	++
Glucose alone	28	++
Calcium glycerate	28	++
Glycerol	26	++
Sodium lactate	26	+
Butyl alcohol	22	++
Sodium butyrate	22	—
Butyl alcohol + Sodium butyrate	22	+
Amyl alcohol	20	++
Glucose + Sodium lactate	19	++
Trimethylene glycol	18	+
Sodium propionate	16	—
Sodium valerate	14	—
Control	2 to 6	—

Colonies transplanted from these tubes soon lost their ability to produce gas, except from glucose. The organism fermenting glucose was a clostridium, apparently of the *welchii-sporogenes* group; the fermentation products were carbon dioxide, hydrogen and volatile acids, the acids after 6 days consisting of 90 per cent acetic and 10 per cent butyric as shown by the Duclaux method.[54]

An unidentified coccus from a sodium propionate tube showed the ability to utilize 75 per cent of the propionic acid in 6 days without producing gas. Gas could be obtained by feeding this coccus, concentrated by centrifuging, to proteolytic clostridia of the *sporogenes* type, but the gas yields were lower than for the direct degradation of propionic acid fed to digested sewage sludge.

Several mixed cultures were obtained from the agar tubes which produced enough gas to merit further study. Two of these were desig-

nated as culture A, composed of a clostridium and a long rod, and culture B, a clostridium and a vibrio. Both of these cultures gave 1:1 ratios of CO_2:CH_4 from glucose and from ethyl alcohol, but only caused a reduction in the amount of oleic acid added when an excess of peptone was added also. Culture B, in the presence of peptone, liquefied agar so completely in 14 days that it would no longer gel even in an ice bath.

Bacterium coli was isolated in several cases. Maassen[120] reports the ability of *B. coli* to utilize propionic acid; this was confirmed by inoculating aerobic tubes of Maassen's medium with a strain of *B. coli* and determining the residual propionic acid at intervals; 60 per cent of the acid was utilized in two weeks. Duplicate anaerobic tubes inoculated with the same strain, however, showed no utilization in 51 days.

The mixed cultures A and B were similarly inoculated into anaerobic tubes of Maassen's medium (which contains 1 per cent peptone) to which propionic or acetic acid had been added; the tubes were analyzed at intervals for 28 days. Volatile acid production from the peptone masked any utilization of the added acid that may have occurred; thus, at the end of 14 days, the following values were found:

	Volatile acid (as acetic) mg. per 100 cc.		
Nutritive added	Culture A	Culture B	Control
Sodium propionate	290.	295.	58.8
Sodium acetate	288.	307.	64.8
Sodium propionate + glucose	302.	278.	60.0
Sodium acetate + glucose	372.	343.	69.6

Only small amounts of gas were produced in these tubes.

Similar quantitative studies were made on our basic medium (without agar) with "ripe" sludge as the inoculum. The results may be summarized thus; acetic or propionic acids (as sodium salts) fed to sludge show a uitilization commencing in 1 to 6 days and continuing to completion in 36 to 52 days; one series of propionic acid (out of the 5) was an exception in that it failed to digest for 44 days and then went rapidly to completion in the next 8 days. Peptone or glucose added along with these acids produced an abrupt increase (doubling) in total volatile acid for the first 6 to 12 days, then utilization of this acid at about the same rate as before.

Nearly 600 tests of this general type were leading into bacteriological ramifications too extensive to follow up in the present chemical study. These tests revealed the following facts:

a. High initial acidities in sludge mixtures can be produced by feeding proteins, peptone, sugars or glycerol. These acidities are the result of biochemical changes brought about mainly by sporeforming anaerobes.

b. Mixed cultures containing proteolytic anaerobes, during active metabolism, can break down the carbon chains of soaps and fats, producing lower acids. Some gas is produced but its origin could not be determined with certainty.

c. The mocroorganisms producing gas from lower fatty acids were not isolated but were shown to occur predominantly in the sludge rather than in the liquor. Thus in two parallel series of 80 cultures each, only two failed to produce gas from acetic and propionic acids when digesting sludge was used for the inoculation, but only three showed gas formation when the supernatant liquor from this sludge was used. In another series of experiments, 36 one-liter quantities of supernatant liquor from sludge digestion tank containing varying amounts of sludge, were fed with propionic acid; the successful gas-producers and the failures are shown in the following summary:

Amount of sludge in liquor, grams per liter	Number of cultures: Digested	Failed
2.58	0	3
3.50	2	3
5.26	0	5
6.60	2	4
9.03	1	2
9.56	6	1
15.63	7	0

d. Certain unidentified anaerobes (cocci) in sludge can utilize acetic and propionic acids (perhaps also others) without gas production. Feeding their dead bodies to proteolytic clostridia produced gas, but the hypothesis that this cycle might be quantitatively important in normal sludge digestion could not be maintained. Thus the CO_2:CH_4 ratio in gas produced by proteolysis of cocci fattened on acetic acid was 2:1 whereas acetic fed directly to sludge yields a 1:1 ratio.

e. Early experiments indicated that carbohydrate was essential for fat degradation and that even anaerobically "fats burn in the flame of carbohydrates;" but this was not supported by later experiments, and cultures A and B, without carbohydrate but with 5 per cent of peptone and oleic broke down 30 per cent of the latter (apparently to acetic) in a week.

f. When a mixture of butyric and acetic acids is fed to "ripe" sludge, butyric disappears more rapidly than acetic, as shown by Duclaux distillations. Propionic acid is erratic, sometimes going much faster than acetic but more often showing a long lag phase of inactivity until 50 to 60 per cent of the acetic has digested. However, when propionic acid does digest, it gives good gas yields as Buswell and Neave[33] have shown.

An attempt was made to determine the possible intermediate substances in gas-production from fats by feeding the suspected substances to nearly "ripe" sewage sludge and noting the change in gas evolution. Sludge which is nearly "ripe" or thoroughly digested continues to produce gas slowly for a week or two during which time the gas-production curve is a straight line of gentle slope. A precursor of methane added to such a sludge should give a sudden jump in gas yield as shown for lactic acid in Figure 2, while a toxic substance or a food available with-

out degradation involving gas production would lessen the gas yield as shown for trimethylene glycol. Furthermore, if a flora were established by feeding butyric acid, and if beta-hydroxybutyric is assumed to be a step in its breakdown, then feeding the latter should give immediate response in gas yield, while feeding some other fermentable material, say lactic acid, should give only a slow increase in yield corresponding to the establishment of another flora. Conversely a flora established on propionic acid, with lactic as the assumed intermediate, might respond immediately to lactic but slowly to beta-hydroxy-butyric acid. No such specificity could be established, and the variety of substances (alcohols,

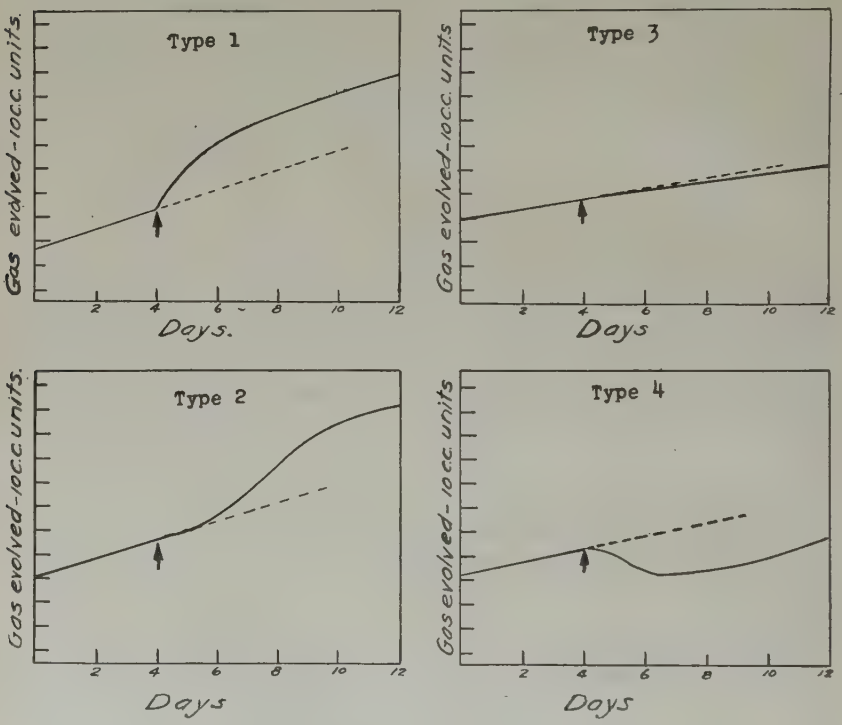


Fig. 2—Types of gas curves in feeding suspected intermediates.

Type 1—Lactic acid, acetaldehyde, ethyl alcohol, glycerol.

Type 2—Propionaldehyde, succinic acid, propyl alcohol, acetone.

Type 3—Malic acid, cinnamic acid.

Type 4—Trimethylene glycol, amino acids, ammonium salts, amines.

Note: Arrow marks the point at which the suspected intermediate was introduced into the slowly gassing sludge.

acids, aldehydes, esters, and ketones) which stimulated gas evolution rendered the method worthless for detecting intermediates. In some cases, in fact, the added substance appeared merely to stimulate gas

production from other materials without yielding gas itself (notably maleic acid). Amino acids (glycocoll, aspartic acid and cystine) and ammonium salts temporarily stopped gas production.

To summarize the question of grease degradation this far, then, it has not been found possible to effect the degradation of soaps and fats excepting in actively fermenting sludge mixtures or in mixed cultures of anaerobes decomposing peptone; in both cases the degradation products of grease are concealed by similar products from other reactions. In sludge digestion, Buswell and Neave[33] have shown that 25 to 40 milligrams of solid nitrogenous matter are liquefied per gram of fatty acid digesting.

In view of this difficulty in following the chemical steps in fat degradation, attention was not turned to the study of lower fatty acids, with the idea that degradation mechanisms applicable to acids of 2 to 5 carbon atoms might be extended to the higher ones; consequently a few concluding remarks may be made about grease digestion before going on to this other topic.

The beta-oxidation mechanism of Knoop[109] is one's first thought in explaining the degradation of long carbon chains. Anaerobically, the following steps would be necessary:

$$R\text{-}CH_2CH_2COOH \longrightarrow R\text{-}CH{=}CHCOOH + H_2$$

$$R\text{-}CH{=}CHCOOH + H_2O \longrightarrow R\text{-}CH(OH)CH_2COOH$$

and by a repetition of this dehydrogenation and water addition, there would result a dihydroxy intermediate (and another hydrogen molecule), which could split off acetic acid:

$$R\text{-}C(OH)_2CH_2COOH \longrightarrow R\text{-}COOH + CH_3COOH.$$

Thus two hydrogen molecules would be eliminated for each acetic acid molecule split off. For the conditions under which the lower acids were studied, this hydrogen would have to be evolved as gas because no other organic molecule is present to act as a hydrogen acceptor; but practically no hydrogen is evolved and consequently beta-oxidation has been rejected as a mechanism for the degradation of lower acids, as will be shown later. However in the actively fermenting sludge mixtures, such as have been found necessary to effect grease degradation, the conditions are more complex and there are three possible ways in which the hydrogen might be utilized: it might be taken up by hydrogen acceptors resulting from the concurrent protein or carbohydrate degradation; it might react with carbon dioxide to give methane, or it might be utilized by some of the microorganisms present in the sludge.

The ability of sludge to irreversibly bind hydrogen is indicated by the recognized, but unexplained, property of sludge to absorb large amounts of hydrogen gas. Söhngen[182] found that hydrogen-carbon dioxide mixtures fed to sludge disappeared in the correct proportion to form methane according to the equation:

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O,$$

and this possible source of methane in sludge digestion gases has been favored by Imhoff[99] and by Bach and Sierp.[8] Randall and Gerard[158]

have shown the reaction to be thermodynamically possible, it having a negative free energy change at 25° of 11,582 calories. On the other hand, there are bacteria in soils and muds which can assimilate hydrogen without methane production,[169] and we have found that only fresh, fairly actively fermenting sludges show hydrogen absorption to any extent:

Nature of Sludge	H_2 (cc.N.T.P.) absorbed in 7 days per 100 cc. sludge
Actively digesting sewage sludge	800-1200
Propionic acid culture	200-300
Old sewage sludge	50-100
Söhngen acetate culture	none

It seems rather probable that hydrogen-utilizing organisms are present in fresh sludge, but die off in more specialized laboratory cultures and are absent in the highly specialized Söhngen cultures (built up by decantation as described later). However, regardless of whether hydrogen is absorbed by hydrogen acceptors, by union with CO_2 or by bacterial assimilation, its absence in sludge gases does not rule out the possibility of beta-oxidation in the degradation of sewage grease.

The increasingly unsaturated nature of sewage grease, as shown by the higher iodine number, during digestion may indicate dehydrogenation of the carbon chains, or it may result from preferential digestion of the saturated chains. At present then, we can only say that while the absence of hydrogen in digestion gases does not prove the absence of beta-oxidation in fat degradation in sludge digestion tanks, another mechanism is not only possible but is proved to apply to methane production from the lower fatty acids.

Lower Fatty Acids

Historical. Turning now to the question of lower fatty acid degradation, more pertinent information is available. The pioneer work on this subject is credited to Hoppe-Seyler[94] who obtained methane from calcium acetate. Into a 600 cc. flask he put 50 cc. of river mud and then filled the flask with river water containing 9.7677 grams of anhydrous calcium acetate. The flask was closed by a rubber stopper, surrounded externally by a mercury seal, and carrying a gas-delivery tube leading to a mercury-filled reservoir; the gases were withdrawn and analyzed at intervals. No hydrogen was found and only a little nitrogen during the early part of the digestion; the ratio of CO_2:CH_4 then became constant at 1:2, indicating the reaction $Ca(C_2H_3O_2)_2 + H_2O = CaCO_3 + CO_2 + 2CH_4$. Determinations of the sludge solids were made in triplicate on the initial sludge, but only once on the final contents:

	Initial Solids		
	Range	Ave.	Final Solids
	gms.	gms.	gms.
Organic matter	0.2860-0.3255	0.3118	0.3385
Ash	2.3485-2.4205	2.3788	2.1782
Calcium		2.473	2.411

The low final ash was attributed to some visible inorganic matter which adhered to the flask walls and was thought to be a deposited silicate. An unexpected 0.1405 gram of sodium carbonate was found in the products, and was attributed to solution of the glass.

While gas analyses were reported to illustrate the constant 1:2 ratio of CO_2:CH_4, the gas volumes were not given, so no balance of the reactants can be calculated. However he emphasized the complete destruction of all the added acetic (in a year's time) and the failure to find any secondary reaction products.

Hoppe-Seyler tried to decrease the amount of inoculating sludge to a point where increase in organic (bacterial) substance could be followed; but small inocula all failed to produce gas. The slow onset of gas production (2 weeks in this experiment) was attributed to the presence of iron oxide and calcium sulfate which supplied oxygen to oxidize the methane to CO_2 and water, thus explaining the high initial CO_2 observed in such experiments. A similar effect had been noted by him in cellulose fermentation in the presence of iron or manganese oxides, of sulfates such as gypsum, or by briefly opening to the air or exposing to sunlight. Accordingly, digestion flasks were not only carefully sealed but were placed in containers lined with black paper.

The same author stated that calcium lactate fermented, at first to CO_2 and hydrogen, but after several months only CO_2 and methane were produced in a 1:2 ratio; he believed that the lactate had been converted into acetate, and then the latter fermented with the normal gas ratio. No analytical data were given.

Mazé[123] fermented acetate in a culture of decaying leaves and isolated a spherical organism, usually in clusters and resembling a large sarcina. Its pure culture did not ferment acetate, but did so when accompanied by either of two sporulating rod forms which also alone could not produce fermentation. Omelianski[141] had noticed this pseudosarcina, and showed that an impure culture of it, added to 0.1 per cent peptone plus 0.5 per cent sodium or calcium butyrate, produced gas in about a month; the gas contained 4 to 8 per cent of CO_2 and 96 to 98 per cent of methane in the ratio of 2:5; the equation was accordingly written:

$$Ca(C_4H_7O_2)_2 + 3H_2O = CaCO_3 + 2CO_2 + 5CH_4.$$

Söhngen[183] extended the studies on fatty acid breakdown to include all the lower acids. His procedure was to place 40 cc. of canal or sewer slime in a 300 cc. erlenmeyer flask and fill it with a medium composed of ordinary water, 100 cc.; K_2HPO_4, 0.05%; NaCl, 0.05%; and calcium salt of the fatty acid, 2%. The temperature was held at about 35°. When the rate of gas evolution began to decrease, he decanted the supernatant liquor and added fresh medium; in this way very active sludges were soon built up, and the cultures could be maintained for a year or more with no change in diet. The sediment became white due to loss of organic matter and deposition of calcium carbonate. The organisms were found to be concentrated in the sediment, and the liquor was almost worthless for inoculating other flasks. Two organisms were described; a rod and a sarcina. The rod was prevalent in cultures

fed with acids other than acetic, and it produced more active fermentations than the sarcina which predominated in acetate cultures. Söhngen found that all of the lower fatty acids with even numbers of carbon atoms were fermented by the same culture, regardless of what acid was used to build up the culture. He expressed the decompositions in the form of equations, such as:

$$Ca(C_2H_3O_2)_2 + H_2O = CaCO_3 + CO_2 + 2CH_4,$$

and his gas ratios were given for the evolved gases only; viz. 1:2 in this case (our ratios refer always to total CO_2 produced, not CO_2 evolved, and for this equation would be 1:1). The ratios reported by Söhngen are:

Formate	2:1	(CO_2:CH_4)
Acetate	1:2	
Butyrate	2:5	
Caproate	3:8	
Caprylate	4:11	
Caprate	5:14	

He was unable to ferment propionic, valeric, heptylic and nonylic acids.

It may be marked in passing that Söhngen's rod-form has not been identified with certainty in cultures examined by us, but a sarcina and a rod departing somewhat from Söhngen's description have been found in our laboratory cultures, in cellulose- fermentation studies which are in progress in this Division, and in separate sludge-digestion tanks at our experimental plant. As Söhngen observed, the sarcina is frequently lime-encrusted and stains only after acid treatment. If the rod-form, which is said to characterize all but acetate fermentations, is shown definitely to be absent in sludge digestion, the inference would be that acetic acid is the only intermediate lower acid in gas production.

In recent years, Coolhaas[46] has reported the thermophilic fermentation of acetate, formate, isobutyrate, oxalate, lactate and gluconate. Propionate and n-butyrate fermented slowly. Acetate gave an average gas composition of 32.8 per cent CO_2 and 66.4 per cent CH_4, and, based on the methane evolved, yields of 96.2 and 97.1 per cent of the theory in two experiments. Formate gave a 94.4 per cent yield of gas according to the equation:

$$2Ca(HCO_2)_2 = CH_4 + CO_2 + 2CaCO_3.$$

Cultures "purified" by long continued feeding with fatty acids were found to ferment cane sugar giving a 93.3 per cent yield of CO_2 and methane in a 1:1 ratio, the equation being:

$$C_{12}H_{22}O_{11} + H_2O = 6CO_2 + 6CH_4.$$

Cultures insufficiently "purified," and occasionally even the old ones, gave a gas composed of CO_2 and hydrogen.

In all of these investigations, a portion of the CO_2 is assumed to deposit as calcium carbonate and a strictly quantitative balance was possible only in terms of methane; that is, the important transforma-

tions in combined oxygen had to be assumed. Accordingly, we have extended these investigations to include a complete balance of the participating substances, and data on fatty acids with an odd number of carbon atoms.

Experimental. Preliminary digestion results on some of the fatty acid salts and other substances have already been reported by Buswell and Neave.[33] These results were obtained by feeding approximately one-gram quantities of the substance in question to one-liter bottles containing 50 to 100 cc. of well-ripened Imhoff tank sludge plus enough domestic sewage to fill the bottle completely. The bottles were closed with rubber stoppers carrying connections to gas collectors filled with saturated brine. The object of these tests was to find how completely the various substances would be decomposed in the normal digestion period of 4 to 8 weeks, and the approximate gas-volumes yielded by their decomposition. Some of the results are shown in Table XI.

TABLE XI

DIGESTION DATA ON FATTY ACIDS AND SOAPS

Substance digested	No. of tests	Yield of CO_2+ CH_4 per gram Grams	Total gas per gram cc.	CH_4 in gas evolved Per cent
Control	1		424	64.3
Na formate	1	0.19	690	48.6
Na acetate	1	0.44	738	52.6
Ca acetate	1	0.65	965	66.1
Na propionate	1	1.08	1667	66.0
Na butyrate	1	0.97	1515	65.6
Na valerate	1	1.00	1407	67.9
Na oleate	3	0.98-1.09	1628 (ave.)	50.5 (ave.)
Ca oleate	1	0.54	942	62.2
Sewage grease	5	0.48-0.51	797-1180	67.7 (ave.)

In these tests, dissolved and combined carbon dioxide in the final bottle contents was not accurately determined; the data have accordingly not been corrected for the control and no attempt has been made to obtain CO_2:CH_4 ratios for the gaseous products.

With the development by C. S. Boruff of this laboratory of an accurate method for determining carbon dioxide in the digestion mixtures, some of the tests were repeated with a view to obtaining gas ratios. Into one-liter brown-glass bottle were put quantities of well-ripened Imhoff tank sludge, representing about 7 grams of dry solids, and 100 cc. of aqueous solution containing the substance to be fermented; then the bottles were filled with domestic sewage and attached to gas collectors. The gas was measured and analyzed from time to time, and finally, after 50 days, the bottle contents were analyzed. Table XII gives the details of these analyses, the following summary presents the values of most importance in the present discussion:

TABLE XII

ANAEROBIC DIGESTION DATA ON BOTTLE TESTS

Using sodium salts of formic to valeric; also oleic and glycerol
(results in grams per bottle unless otherwise designated)

	Initial for all bottles	Control 1	CH_2O_2 2	$C_2H_4O_2$ 3	$C_3H_6O_2$ 4	$C_4H_8O_2$ 5	$C_5H_{10}O_2$ 6	Oleic 7	Glycerol 8
Sludge: Total Dry solids	7.2	6.9	6.7	6.9	6.8	6.6	6.6	6.6	6.5
Organic matter	3.3	3.1	2.7	2.7	2.8	2.7	2.7	2.7	2.6
Liquor: Total solids	0.80	0.95	1.72	1.81	1.39	1.43	1.42	1.22	0.89
Volatile acid: added	0.07	0.07	0.75	0.80	0.84	0.70	0.72	0.87	1.08
recovered	.....	.11	.11	.13	.11	.12	.11	.10	.11
digested	.....	.....	.64	.67	.73	.58	.61	.77	.97
Total nitrogen: sludge	.186	.157	.141	.145	.146	.137	.144	.147	.141
liquor	.052	.088	.094	.088	.094	.103	.096	.087	.089
Sum	.238	.245	.235	.233	.240	.240	.240	.234	.230
pH of Liquor	7.4	7.0	7.6	7.6	7.6	.....	.....	.....	7.2
Total cc. of gas: CO_2	.....	50.4	142.	264.	259.	218.	205.	207.	441.
(2-8 corrected CH_4	.....	225.	64.	233.	396.	305.	341.	417.	409.
for control) H_2	.....	11.1	0.0	2.2	0.0	18.4	2.2	18.3	16.6

Substance fed	Free acid digested Grams	CO_2+CH_4 produced	Ratio of CO_2:CH_4 Found	Theory*
Control	———	0.158	1:4.5	———
Na formate	0.64	(.329)	(1:0.4)	1:0.33
Na acetate	.67	.691	1:0.9	1:1
Na propionate	.73	.795	1:1.5	1:1.4
Na butyrate	.58	.649	1:1.4	1:1.66
Na valerate	.61	.649	1:1.7	1:1.85
Na oleate	.77	.706	1:2.	1:2.3
Glycerol	.97	1.168	1:0.9	.....

*Theoretical ratios are based on the mechanism described later.

The grams of gas produced are corrected for the control. The gas data on sodium formate are doubtful because during the late stages of its digestion the green color of *B. pyocyaneus* was noticed in the bottle; the nitrogen in the gas increased to 34.7 per cent and the carbon dioxide decreased to 7.2 per cent. Autotrophic utilization of CO_2 by this organism apparently was in progress here as in some of the nitrogen studies previously mentioned.

Taken by themselves, these bottle tests are not sufficient basis for the formulation of a digestion mechanism; they are cited merely as corroborative evidence for the mechanism to be described later. The principal sources of error in them are the uncertain changes in the quantity and character of the inoculating sludge during digestion, and the high ratio of this sludge to the organic food decomposed. In handling a heterogenous solid mixture like sewage sludge, sampling errors are large, and only the mean of a series of tests can be given much weight. Another source of error is the uncertainty in the truly representative nature of the control; the addition of fatty acids to a sludge may promote the digestion of some of its components; the resultant loss in material may even be replaced by humus-like products from fatty acid metabolism or by increase in the quantity of bacterial protoplasm. Gas ratios accordingly become uncertain even though corrections based on an unfed control be applied. The magnitude of this uncertainty can be seen in results taken from Table XII. If for these bottles the total solids of the sludge are added to those in the liquor, the total solid contents can be compared. However, the final solids in bottles fed with sodium salts must be corrected for this addition of inorganic matter; we have assumed that all of the sodium is weighed in the final determinations as sodium carbonate, and the calculated weight of sodium carbonate, corresponding to the weight of salt fed, has been deducted from the final total solids, thus:

	Initial	Final Analyses 1	2	3	4	5	6	7	8
Sludge solids	7.2	6.9	6.7	6.9	6.8	6.6	6.6	6.6	6.5
Liquor solids	0.80	0.95	1.72	1.81	1.39	1.43	1.42	1.22	0.89
Sum	8.00	7.85	8.42	8.71	8.19	8.03	8.02	7.82	7.39
Na_2CO_3 correction	.....	.....	0.78	0.64	0.44	0.38	0.34	0.17	.....
Corrected solids	8.00	7.85	7.64	8.07	7.75	7.65	7.68	7.65	7.39

The 0.15 gram decrease in total solids for the control (No. 1) compares favorably with the weight of gas which this bottle produced; viz, 0.158 gram. But with the exception of the acetate (No. 3), the other values suggest an enhanced digestion of solid material as a result of feeding, this being particularly marked in the case of glycerol (No. 8).

These considerations made apparent the changes in procedure necessary to obtain reliable gas ratios from which a degradation mechanism could be inferred,—either the quantity of inoculating sludge had to be greatly reduced, or the quantity of acid fed had to be greatly increased. The previous studies on pure-culture isolation had shown that gas-producing organisms occurred almost only in the sludge and that if the quantity of inoculating sludge were reduced to a low value, comparatively few successful inoculations resulted. This minimum inoculating ratio cannot be defined quantitatively; it varies widely with the nature of the sludge in question. Since a record has been kept in nearly all cases of the nature of the inoculum for different series of digestion tests, the following summary has been compiled for the types of inoculum available at our experimental plant. The summary lists merely the numbers of successful and unsuccessful inoculations as judged by gas production from grease, soaps or lower fatty acids:

	Successful	Failed
Old sludge (in lab. ½ to 2 months)	79	76
Fresh "active" sludge (Imhoff tank)	33	2
Tank liquor + various sludges	27	13
Liquor alone (digestion tanks)	47*	54
Nidus tank solids	15†	6

*43 of these liquors were collected just after circulating and contained sludge.

†These all started very slowly.

The other alternative, that of increasing the fatty acid fed, was applied to a series of 36 one-liter digestion tests on propionic and butyric acids. Of these, 18 failed to digest and 2 had to be discarded on account of *B. pyocyaneus* growth. The successful bottles contained 4 to 9 grams of organic solids in the inoculum and were fed with 5 to 10 gram quantities of the sodium or calcium salts. The detailed analytical results are presented in Tables XIII, XIV and XV, but for convenience they may be summarized as follows:

Salt fed	Acid digested Grams	Gas yield per cent of theory CO_2	CH_4	CO_2:CH_4 ratio	Assigned weight for mean
Na propionate	4.14	120.	94.6	(1:1.1)	1
Na propionate	8.60	106.	88.2	(1:1.2)	2
Na propionate	4.49	115.	115.	(1:1.4)	1
Na propionate	8.94	86.2	79.1	(1:1.3)	2
Na propionate	2.12	93.7	87.0	1:1.6	1
Na propionate	4.65	105.	93.4	1:1.44	5
Na propionate	9.13	96.2	82.5	1:1.32	5
Ca propionate	3.43	91.7	109.	1:2.03	2
Weighted means		100.1	90.9	1:1.42	
Na butyrate	4.92	88.6	71.2	1:1.68	3
Ca butyrate	4.89	71.0	90.7	1:2.07	1
Ca butyrate	9.77	90.7	97.5	1:1.77	2
Weighted means		86.4	83.2	1:1.77	

In this summary, the percentage yield of gas is calculated both for the CO_2 and for the CH_4; the theoretical yields of these gases,

TABLE XIII

ANAEROBIC DIGESTION OF SODIUM PROPIONATE

(Results in grams per bottle)

	Initial without acid	5.67 gms. sodium salt	11.35 gms. sodium salt	Control bottle
First Series				
CO_2 in bottle contents — Initial	1.74	1.74	1.74	1.74
CO_2 in bottle contents — Final		4.60	7.04	2.27
Gas produced* — CH_4		1.476	2.868	(failed
Gas produced* — CO_2		3.686	6.754	to gas)
Gas produced* — H_2		.009	.021	
Total solids in sludge — Initial	6.60	6.60	6.60	6.60
Total solids in sludge — Final		9.98	12.17	6.76
Ash in sludge — Initial	3.10	3.10	3.10	3.10
Ash in sludge — Final		6.13	8.82	3.45
Organic solids — Initial	3.50	3.50	3.50	3.50
Organic solids — Final		3.85	3.35	3.31
Volatile acids — Initial	0.01	4.38	8.76	0.01
Volatile acids — Final		0.24	0.16	0.18
pH of liquor	7.2	7.7	7.8	7.4
Digestion period, days		43	76	43

Second Series

CO_2 in bottle contents	Initial	1.33	1.33	1.33	1.33
	Final		4.16	5.85	1.57
Gas produced* CH_4			1.960	2.673	(failed
CO_2			3.850	5.724	to gas)
H_2			.010	.012	
Total solids in sludge	Initial	9.73	9.73	9.73	9.73
	Final		12.09	13.71	8.18
Ash in sludge	Initial	4.69	4.69	4.69	4.69
	Final		6.82	9.14	4.50
Organic solids	Initial	5.04	5.04	5.04	5.04
	Final		5.27	4.57	3.68
Volatile acids	Initial	0.33	4.70	9.08	0.33
	Final		0.21	0.14	0.15
pH of liquor		7.4	7.6	7.8	7.6
Digestion period, days			47	74	47

*Weights of gases are corrected for the dissolved and chemically bound gases in the solution.

TABLE XIV

SODIUM PROPIONATE AND LACTATE DIGESTION DATA

(Results in grams per bottle)

		Initial mixture	2.28 gm. sodium propionate	5.71 gm. sodium propionate	11.42 gm. sodium propionate	Control	7.71 gm. sodium lactate
CO_2 in bottle contents	Initial	1.22	1.25	1.26	1.28	1.28	2.16
	Final		2.36	4.12	6.54	1.45	4.35
Gas produced* CH_4			1.229	2.177	3.380	0.533	2.165
CO_2			2.024	4.173	7.073	0.553	4.438
H_2			.007	.009	.017	.002	.009
Total solids in sludge	Initial	9.40	9.59	9.68	9.87	9.87	15.63
	Final		9.70	12.44	16.88	9.41	19.07
Ash in sludge	Initial	4.37	4.46	4.50	4.59	4.59	6.86
	Final		5.82	7.55	10.92	5.64	11.09
Organic solids	Initial	5.03	5.13	5.18	5.28	5.28	8.77
	Final		3.88	4.89	5.96	3.77	7.98
Volatile acids	Initial	0.45	2.22	4.86	9.28	0.47	6.47
	Final		0.10	0.21	0.15	0.11	0.16
pH of liquor		7.2	7.6	7.4	7.8	7.6	7.6
Digestion period, days			38	60	71	38	55

NOTE: Control for the sodium lactate bottle is the one listed in Table XV.
*Corrected for the control and for dissolved gases.

TABLE XV

CALCIUM PROPIONATE, SODIUM BUTYRATE AND CALCIUM BUTYRATE DIGESTION DATA

(Results in grams per bottle)

		Initial mixture	4.27 gm. calcium propionate	6.01 gm. sodium butyrate	5.85 gm. calcium butyrate	11.70 gm. calcium butyrate	Control
CO_2 in bottle contents	Initial	2.16	2.16	2.16	2.16	2.16	2.16
	Final		3.07	4.42	2.96	2.09	2.10
Gas produced*	CH_4		2.330	2.518	2.507	4.322	0.930
	CO_2		3.185	4.114	3.327	6.649	.846
	H_2		.022	.021	.014	.026	.004
Total solids in sludge	Initial	15.63	15.63	15.63	15.63	15.63	15.63
	Final		18.09	18.58	16.29	17.93	15.72
Ash in sludge	Initial	6.86	6.86	6.86	6.86	6.86	6.86
	Final		11.83	12.43	8.67	11.81	9.59
Organic solids	Initial	8.77	8.77	8.77	8.77	8.77	8.77
	Final		6.26	6.15	7.62	6.12	6.13
Volatile acids	Initial	0.27	3.67	5.08	5.08	9.89	0.27
	Final		0.24	0.16	0.19	0.12	0.13
pH of liquor		6.8	7.0	7.1	6.8	7.2	6.8
Digestion period, days			55	55	40	60	41

*Corrected for the control and dissolved gases.

based on the weight of acid digested, were calculated from the following equations, since the subsequent discussion will show that these represent the degradation mechanism:

Propionic: $4C_3H_6O_2 + 2H_2O = 5CO_2 + 7CH_4$

Butyric: $2C_4H_8O_2 + 2H_2O = 3CO_2 + 5CH_4$

It will be noted that the theoretical CO_2:CH_4 ratio for propionic is 5:7 or 1:1.40 by volume, which compares favorably with the weighted mean in the summary; for butyric it is 3:5 or 1:1.66, the value actually found being somewhat high. The propionic gas ratios in parentheses represent bottles uncorrected for the controls (the controls failed to produce gas). In weighting the means, the weights have been assigned according to general reliability of the analytical data, a low weight indicating imperfect checks or some other uncertainty in sampling or analytical precision. However, the data are all presented in the tables for those who may prefer some other treatment of them. Attention is called also to the fact that the CO_2 yield exceeds that of the methane, and that small amounts of hydrogen are present in the gases; it will be necessary to refer back to these facts in the later discussion.

The limitations of the bottle-type of digestion experiment are:

(a). Sludge to the amount of 5 to 10 grams per liter seems to be necessary to ensure digestion; otherwise days of analytical work on

the initial set-up, and weeks of carefully nursing a feeble gas evolution, are apt to be wasted.

(b). Quantities of sodium salts exceeding about 10 grams per liter either did not ferment readily, or stopped before digestion was complete on account of the high concentration of residual sodium bicarbonate. Feeding calcium salts avoids the high alkalinity, because calcium carbonate precipitates, but, with the exception of the lower ones, they are of limited solubility.

The above difficulties have been overcome by the construction of an eight-liter reaction vessel of galvanized iron, cylindrical in form but tapering to a cone beneath and provided on top with a water-sealed, gas-collecting dome whose outlet leads to a gasometer. Additional connections permit the withdrawal of supernatant liquor or the feeding of fatty acid salts without admitting air into the system. A diagram of the equipment is shown in Figure 3. In such an apparatus 25 to 50 grams of acid per week can be metabolized, and the experiment continued until the organic matter in the inoculum represents a negligible percentage of the total metabolism. To avoid lethal osmotic effects and hydroxyl-ion concentrations, either the calcium salt, or the sodium salt alternated with free acid, is fed in daily rations in the form of Söhngen medium, the displaced supernatant liquor being saved for analysis. The details of one feeding experiment on calcium acetate will illustrate the technique employed.

The vessel was filled with 6800 cc. of liquor from a sludge digestion tank, 800 cc. of sludge from the same tank and 200 cc. of Söhngen's medium. An extra portion of the liquor-sludge mixture (without the medium) was reserved for analysis. The daily rations of Söhngen medium were prepared just before use by diluting a stock calcium acetate solution (approximately 10%) with tap water; the preparation of all Söhngen medium involved measuring out 40 cc. of the stock solution, adding 5 cc. of a solution containing 0.1 gram each of NaCl and K_2HPO_4, and diluting to 200 cc. with tap water, or for part of the run, to 100 cc. with water and then 100 cc. of supernatant liquor from the reaction vessel added. Feeding was accomplished by withdrawing 200 cc. of supernatant liquor and replacing it with 200 cc. of medium introduced near the bottom of the tank. During most of the experimental period, the tank was fed twice daily at twelve-hour intervals. The run lasted for 35 days, during which 159.7 grams of acetic as calcium acetate had been fed, and about 17 liters of CO_2 and 39 liters of CH_4 had been evolved.

At the end of the run and before the tank contents had been disturbed, duplicate determinations of dissolved CO_2 were made on 500 cc. portions of the supernatant liquor. The tank was then emptied and the entire contents filtered on a buchner funnel. The sludge cake

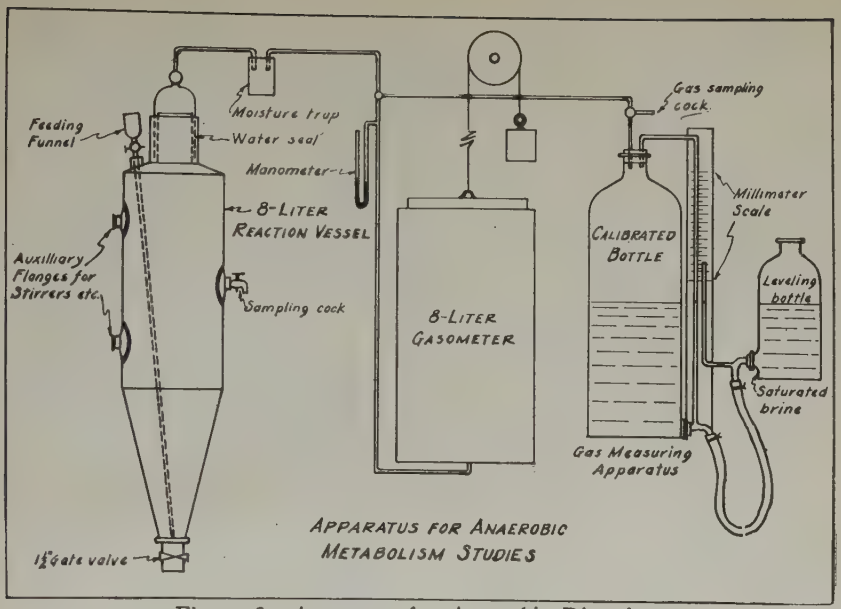


Figure 3.—Apparatus for Anaerobic Digestions.

was dried and analyzed separately. Considerable carbonate and phosphate scale remained adhering to the inner walls of the tank; this was dissolved in dilute acetic acid, and determinations of calcium and phosphate made on these acetic washes so that the CO_2 in the scale could be calculated. The analytical data in Tables XVI, XVII and XVIII furnish the basis for the subsequent calculations and illustrate the method followed in these tank runs.

The final balance of the reactants may be summarized thus:

Total acetic acid added was 159.69 grams, and that undecomposed at the end of 45.51 grams, giving a total metabolism of 114.18 grams; this quantity of acetic acid should yield 30.489 grams of CH_4 and 83.691 grams of CO_2. The actual yield of CH_4 was 28.078 grams, or 92.9 per cent of the theoretical. A total of 82.968 grams of CO_2 was found in the final products, but of this, 3.072 grams were added as initial sludge and 0.679 gram as tap water; making this correction, the acetic acid actually yielded 79.217 grams of CO_2 or 94.7 per cent of the theoretical. In addition to the CH_4 and CO_2, there was produced also 0.106 gram of hydrogen. Balances of the other reactants are presented in Table XVIII.

Attention is called to the facts that neither the CH_4 nor the CO_2 yield attained 100 per cent, but the percentage yield of CO_2 exceeded that of CH_4; also a small amount of hydrogen was evolved. These

TABLE XVI

Daily Data Sheet

CALCIUM ACETATE DIGESTION

Days	Feed in grams as Acetic	Feed in grams as Ca	Feed in grams Tap water	Feed in grams NaCl	Feed in grams K_2HPO_4	Overflow liquor cc.	Temperature Tank	Temperature Room	Barometer mm.	Gas produced cc. N.T.P.	Gas analysis Per cent CO_2	H_2	CH_4	N_2	cc. at N.T.P. CO_2	cc. at N.T.P. CH_4	cc. at N.T.P. H_2	Remarks
0	3.048	0.976	155	0.1	0.1	200	....	....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
1	3.048	0.976	155	0.1	0.1	200	25	25	743	430								
2	3.048	0.976	155	0.1	0.1	200	26	26	738	1600	23.2	0.5	72.6	3.7	471	1474	10.1	Single daily feeding
3	3.048	0.976	155	0.1	0.1	200	24	23	738	1425	19.4	1.3	74.5	4.8	249	1062	18.5	
4	3.048	0.976	155	0.1	0.1	200	23	24	743	1080								
5	3.048	0.976	155	0.1	0.1	200	26	23	748	910								
6	3.048	0.976	155	0.1	0.1	200	26	24	749	1640								
7	3.048	0.976	155	0.1	0.1	200	26	24	746	1980	18.8	1.6	66.1	13.5	1440	5076	122.9	
8	3.048	0.976	155	0.1	0.1	200	26	26	743	2070								
9	3.048	0.976	155	0.1	0.1	200	25	27	744	1870								
10	3.048	0.976	155	0.1	0.1	200	25	18	750	2025								
11	2.868	0.908	155	0.1	0.1	200	26	26	748	1710	24.4	1.4	66.7	7.5	1785	4879	102.4	
12	2.868	0.908	155	0.1	0.1	200	26	26	748	1710								
13	2.868	0.908	155	0.1	0.1	200	25	25	741	1620	25.1	1.6	67.4	5.9	407	1092	25.9	
14	2.868	0.908	155	0.1	0.1	200	25	21	738	1485								
15	2.868	0.908	155	0.1	0.1	200	24	18	738	1500	22.9	2.3	62.3	12.5	1038	2825	103.2	
16	5.736	1.816	310	0.2	0.2	400	24	22	744	1550								
17	5.736	1.816	110	0.2	0.2	200	24	19	754	2420	24.8	2.0	61.4	11.8	600	1486	48.4	Fed twice daily. Concentration of feed doubled
18	5.736	1.816	110	0.2	0.2	200	23	18	753	2600								
19	5.736	1.816	110	0.2	0.2	200	25	22	746	2880	26.5	3.1	56.8	13.6	1452	3113	169.9	
20	5.736	1.816	110	0.2	0.2	200	25	22	745	3403	33.1	1.6	61.5	3.8	1126	2093	54.4	
21	5.736	1.816	110	0.2	0.2	200	25	25	746	3298	32.3	2.7	50.5	14.5	1065	1665	89.0	
22	5.736	1.816	110	0.2	0.2	200	25	25	738	2884	30.0	1.8	64.1	4.1	865	1849	51.9	
23	9.016	2.856	110	0.2	0.2	200	26	25	743	2817	28.7	1.8	58.6	10.9	808	1651	50.7	
24	9.016	2.856	110	0.2	0.2	200	26	25	743	2943	28.9	1.7	60.4	9.0	850	1778	64.6	
25	7.416	2.348	110	0.2	0.2	200	26	25	743	2853	28.8	1.8	59.8	9.6	822	1706	51.3	
26	5.816	1.840	110	0.2	0.2	200	26	26	737	2501	30.0	3.4	62.7	3.9	750	1568	85.0	
27	5.816	1.840	110	0.2	0.2	200	26	26	741	2292	30.5	1.4	63.3	4.8	699	1451	32.1	
28	5.816	1.840	110	0.2	0.2	200	24	24	736	1437								
29	2.908	0.920	55	0.1	0.1	100	25	19	743	1124	28.8	1.3	61.6	8.3	738	1578	33.2	No evening feeding
30	.....	.....	.....	.....	.....	.....	25	24	753	1156	29.5	1.0	63.3	6.2	341	732	11.6	No feeding
31	6.468	.....	100	.....	.....	100	25	24	755	855	29.9	1.7	62.9	5.5	256	538	14.5	Acetic acid fed
32	6.468	.....	100	.....	.....	100	25	24	750	1006								
33	6.466	.....	100	.....	.....	100	26	27	742	837	43.2	1.2	52.7	2.9	780	971	22.1	Acetic acid fed
34	6.468	.....	100	.....	.....	100	26	26	747	571	59.2	1.2	37.6	2.0	690	215	6.8	Acetic acid fed
35	.....	.....	.....	.....	.....	.....	25	25	746	774*	59.4	1.2	37.5	1.9	460	290	9.3	Run stopped
Totals																		
	159.696	42.488	4565	4.3	4.3	6300									16,696	39,092	1177.8	
											dissolved.....					107	1.7	
																39,199	1179.5	

*Includes 310 cc. in gas system at end of run.

TABLE XVII

ANALYTICAL DATA ON CALCIUM ACETATE DIGESTION

(Results in grams in the tank contents)

	Acetic acid	Total solids	Ash	CO_2	Ca	P_2O_5	N_2
7.8 liters Initial inoculum (without Ca acetate)	1.86	63.1	25.3	3.072	3.516	1.2836	4.043
100.8 grams (dry) Final sludge	.....	100.8	62.4	20.202	20.563	2.275	1.2822
7.7 liters Final liquor	38.27	60.21	21.9	8.707	12.751	0.1406	1.0079
Acetic solution of final scale:							
Insoluble sludge	.....	7.35	3.55	0.141*	0.2238	0.1700	0.2644
8.23 liters wash solution	.....	.....	.....	8.460*	7.822	0.2337	.....
6.3 liters overflow liquor composite	7.24	25.5	17.8	12.644	5.809	0.2157	1.9228
4.565 liters tap water added	.....	1.67	.....	0.679	0.293	.....	0.0114

*Calculated from Ca present in excess of the P_2O_5.

TABLE XVIII

CALCIUM ACETATE DIGESTION DATA

		Total		Organic
Solids added:	Initial in tank	63.1		37.8
	Calcium acetate	167.8		159.7
	Acetic acid (free)	34.4		
	NaCl + K_2HPO_4	8.6		
	Tap water solids	1.7		
		275.6		197.5
Solids, final:	Tank liquor	60.2		38.3
	Tank sludge	100.8		38.4
	Tank scale	27.1		3.8
	Overflow	25.5		14.9
	CO_2 + CH_4 + H_2	107.4		107.4
		321.0		202.8
Calcium:	Added—Initial sludge	3.516	Final—Liquor	12.751
	As acetate	42.488	Sludge	20.563
	As tap water	.293	Scale	8.046
			Overflow	5.809
		46.297		47.169
Phosphate:	Added—Sludge	1.2836	Final—Liquor	0.1406
	K_2HPO_4	1.7528	Sludge	2.275
			Scale	.4037
		3.0364	Overflow	.2157
				3.0350

Nitrogen:	Added—Sludge	4.043	Final—Liquor	1.0079
	Tap water	.0114	Sludge	1.2822
			Scale	.2644
		4.0544	Overflow	1.9228
				4.4773

NOTE: The above data are total grams in the tank. Calcium is calculated as Ca, phosphates as P_2O_5 and nitrogen as N.

facts will be recalled in the discussion of the degradation mechanism, because the excess CO_2 and the hydrogen appear to be indications of the anabolic phase of metabolism rather than of the respiratory degradation of acetic to furnish energy.

In this experiment, the inoculating sludge contained an initial 37.8 grams of organic matter, and a final 38.4 grams. Apparently then the inoculum furnished little, if any, gas; but even if it had all gasified, the ratio of acetic acid to organic solids is still 3:1; thus the degree of accuracy is far superior to that attained in bottle test.

A second tank experiment on calcium acetate was run primarily to study the effect of varying conditions and technique. A total of 75.08 grams of acetic acid were metabolized, and yielded 89.7 per cent of the theoretical CH_4 and 92.1 per cent of the theoretical CO_2; in addition also 0.053 gram of hydrogen was evolved. The analytical data for this experiment are presented in Table XIX. Here again the excess yield of CO_2 and the production of a little hydrogen are noted. The gas ratios obtained from these acetate digestions are:

1st run 1:0.975 for CO_2:CH_4

2nd run 1:0.974

In these runs, the combined effects of calcium carbonate deposition and carbon dioxide evolution maintainel the pH of the tank contents between 6.7 and 7.2 during the experiment. But the deposition of an adherent carbonate-phosphate scale on the inner tank walls made recovery of all the final products somewhat laborious. Accordingly in later runs the sodium salt was fed until a desirable concentration of sodium bicarbonate had accumulated and then only the free acid fed; this free acid liberated an equivalent amount of carbon dioxide during its neutralization, then the resultant sodium salt digested and

TABLE XIX

CALCIUM ACETATE DIGESTION DATA

Total acetic fed	150.3	CH_4 produced	17.97 gm.
Acetic recovered in tank	75.05	CO_2 produced	50.707
		H_2 produced	.053
Acetic metabolized	75.25		

Total solids:	Added—Initial sludge	64.0	Final—Liquor	90.6
	As acetate	144.0	Sludge	90.6
	As acetic acid	41.3	Scale	5.4
	$K_2HPO_4 + NaCl$	6.6	Overflow	39.0
	Tap water	1.3	Gas	57.3
	Sewage	0.3		
		257.5		282.9
Calcium:	Added—Sludge	3.895	Final—Liquor	17.043
	Acetate and tap water	32.206	Sludge	11.216
		36.101	Scale	2.122
			Overflow	5.094
				35.475
Phosphate:	Added—Sludge	1.0830	Final—Liquor	0.0742
	K_2HPO_4	1.7215	Sludge	2.4216
		2.8045	Scale	.1382
			Overflow	.2251
				2.8591

NOTE: Calcium acetate, in hyrolyzing, adds on one molecule of water which appears in the final products but not in the solids added; this water of hydration amounts to 11.2 grams in this run and to 17.1 grams for Table XVIII, and for a strict total solids balance these values should be added to the weights of total solids introduced into the system.

regenerated the bicarbonate by the time the next twelve-hour feed was added. The desirable bicarbonate concentration has been calculated from the partial pressure of carbon dioxide which should theoretically be found in the gases and from the assumed optimum pH of 7.0. Thus, propionic acid digests according to the equation:

$$4\ C_3\ H_6O_2 + 2\ H_2O = 5\ CO_2 + 7\ CH_4.$$

The evolved gas should contain five-twelfths, or about 41 per cent, of carbon dioxide. The calculation then is:

At 26° and 41% partial pressure, a liter of water dissolves 302.6 cc. = 0.598 gram = 0.0136 mole of CO_2.

Since $[H^+]\ [HCO^-_3] = 3 \times 10^{-7}$, then at pH 7.0,

$$[HCO^-_3] = \frac{[CO_2 + H_2CO_3]}{1 \times 10^{-7}} = \frac{(0.0136)\ (3 \times 10^{-7})}{1 \times 10^{-7}} = 0.0408$$

Assuming 85 per cent ionization of $NaHCO_3$, then 0.0408 HCO^-_3 = 0.0662 $NaHCO_3$ = 5.6 grams per liter, and this can result from about 6.4 grams per liter of initial sodium propionate.

While this value is only approximate, and takes no account of the sodium propionate-propionic acid equilibrium, the method has actually yielded a very satisfactory pH control.

A propionate run, using this system of buffering, yielded an average CO_2:CH_4 ratio in the evolved gas of 5:7.08, while the theoretical is 5:7, and daily records of the pH showed a range of 6.9 to 7.2. The run in question metabolized 130.06 grams of propionic acid in 59 days and yielded 98.7 per cent of the theoretical methane and 99.6 per cent of the theoretical carbon dioxide; in addition 0.243 gram of hydrogen was evolved. The final CO_2:CH_4 ratio was 5.05:7.

The balances pertaining to this run are shown in Table XX and some of the items appearing therein require an explanation. The feeding of the sodium salts like those of calcium, leads to a simple hydrolysis to the free acid and the formation of sodium bicarbonate

TABLE XX

SUMMARY OF PROPIONIC ACID RUN

Propionic acid metabolized 130.06 grams
CH_4 produced 48.587 grams = 98.7 per cent of theoretical
CO_2 produced 96.320 grams = 99.6 per cent of theoretical
H_2 produced 0.243 gram
CO_2:CH_4 ratio 5.05:7

Solids Balance

	Total solids		Total solids
Added as sludge	53.06 gm.	Recovered as sludge	39.36 gm.
Na as Na_2CO_3	35.84	in liquor	37.79
Tap water	1.51	in overflow	32.90
As sludge	3.00	as propionic	38.42
$NaCl + K_2HPO_4$	2.60	as gas	145.15
K_2HPO_4			
$(NH_4)_2HPO_4$	0.04		
H_3PO_4	0.02		293.62
$(NH_4)_2CO_3$	1.3	Correction for Fe_2O_3	—4.93
	97.37		288.69
As propionic	168.48	Correction for H_2O of oxid. of the prop.	—15.82
Total Solids Added	265.85		272.87
		Correction for H_2O of hydrol. of Na prop.	—6.08
		Total Solids Recovered	266.79

	Organic matter		Organic matter
Added as sludge	42.66 gm.	Recovered as sludge	26.14 gm.
Added as sludge	2.41	in liquor	16.40
Added as propionic	168.48	in overflow	11.84
		as propionic	38.42
Organic Solids Added	213.55	as gas	145.15
			237.95
		Correction for H_2O of oxid. of Propionic	—15.82
		Organic Solids Recovered	222.13

Nitrogen Balance

	N		N
Added as sludge	0.441 gm.	Recovered as sludge	0.1132 gm.
as NH_4 prop.	.1485	in liquor	.4669
as $(NH_4)_2HPO_4$	.0078	in overflow	.0394
as tap water	.0285	in samples*	.0254
as sludge	.0265		
	.6523		.6449

*Occasional samples for volatile acids, NH_3, P_2O_5 etc.

which is weighed in the final solids as the carbonate; accordingly a quantity of water according to the equation,

$$CH_3CH_2CO_2Na + H_2O \rightleftharpoons CH_3CH_2CO_2H + NaOH$$

must be deducted from the final total solids. In the present case, this amount is 6.08 grams.

In addition to the water of hydrolysis, propionic acid degradation involves an oxidation by water according to the equation:

$$4\ C_3H_6O_2 + 2\ H_2O = 5\ CO_2 + 7\ CH_4.$$

This water of oxidation appears in the products, but not in the added materials, and must be deducted in striking the balance; for the metabolism of 130 grams of acid, it amounts to 15.82 grams.

A certain amount of iron goes into solution from the tank walls; this has been determined and the resultant 4.93 gram correction applied to the final total solids.

The almost quantitative conversion in these methanic fermentations is well illustrated by this run. The methane produced accounts for 128.37 grams of the total 130.06 grams of acid metabolized, leaving a residue of 1.69 grams to be accounted for; of this, the excess carbon dioxide and hydrogen together account for 1.41 grams. The present accuracy of the balances does not warrant closer dissection of the residuals.

Enough results are now available to indicate the underlying principles governing the method of decomposition. To preface the discus-

sion of mechanisms, however, a brief resumé is given of the general characteristics of anaerobic organisms, if for no other reason than to justify the emphasis later placed on transformations of combined oxygen.

It may be noted in passing that the success achieved with the 8-liter digestion tank for experiments of this type has led to a continuation of the work on higher fatty acids not only to obtain more precise gas ratios for additional support of the respiratory mechanism, but also to investigate the anabolic phase or whatever series of reactions is responsible for hydrogen production.

The Mechanism of Anaerobic Oxidations

As a background for a discussion of the mechanism of anaerobic oxidation, the environmental conditions necessary for the propagation of obligate anaerobes will be reviewed briefly.

By definition, the obligate anaerobes are those microorganisms which are intolerant to elementary oxygen, but the inhibitory and lethal effects of this gas on them have not been entirely explained. Of the theories which have been advanced, none explains completely the diversified behavior of strict anaerobes as revealed by the extensive cultural and physiological study which has been accorded these organisms.

Since February 25, 1861, when Pasteur[146] announced to the French Academy his discovery of "infusorian animalcules" which lived and effected fermentations in the absence of gaseous oxygen, speculation has been rife as to whether even the strictest anaerobes do not require traces of free oxygen. These organisms clearly menaced the doctrine of "no life without oxygen" which had sprung up following the classical work of Lavoisier on this gas. Cultural studies, even in sealed glass globes, were questioned,[80] because of the uncertainty in removing oxygen dissolved in the medium and absorbed on the inner walls. The diffusion of gases through rubber stoppers or tubing ruled out such apparatus entirely. Very delicate chemical tests for free oxygen were developed, and a bitter conflict arose[80,114,138] to prove or disprove the free oxygen requirement of anaerobes. Beijerinck[18] entered the conflict as a peacemaker with the naive suggestion that even strict anaerobes required some free oxygen, but that the amount was too small for detection by any known chemical means; hence they should be called "microaerophiles" and not anaerobes. However, Kürsteiner[113] has been able to carry anaerobes through sixteen inoculations, with the complete exclusion of air in a sealed apparatus, with no loss of activity; he used luminous bacteria as an indicator of anaerobiosis, and Harvey's[85] studies on bioluminescence have shown that these organisms require only one part of oxygen in 3.7×10^9 parts of water to emit light. In addition, the studies of Clark[42] and his associates have permitted the chemical criteria of anaerobiosis (reduction of dyestuffs, etc.) to be translated into terms of equilibrium pressures of oxygen; thus, when the indigo carmine used by Pasteur is 80 per cent reduced by bacteria, the equilibrium oxygen pressure is 10^{-36} atmosphere, or

less than one molecule of oxygen in 10^{13} liters of culture. As Clark[41] points out "To speak colloquially, that is *some* anaerobiosis." Nevertheless, the old postulate of indispensability of free oxygen persists to some extent, particularly in the medical literature and in some textbooks.[58]

This persistence is attributable in part to the anomalies encountered in cultural studies of anaerobes. Thus it is difficult to imagine the nonparticipation of oxygen in a culture of strict anaerobes open to the air, but containing some added turbidity, glass-wool, cotton, beads, coal, iron, animal tissue, or one of a variety of substances[79,86,216] which will permit their growth in open vessels. This phenomenon of inert substances permitting the growth of anaerobes under aerobic conditions still blossoms forth as new discovery now and then in the medical journals[2,53] in spite of its 22 years of age. The effect of glass beads, etc., in cultures can most logically be attributed to the provision of a nidus for the organisms and the minimizing of oxygen transport by retarding convection stirring.

A vast literature has grown up around the use of fresh tissue or symbiotic aerobes in open culture vessels. The work has shown the necessity for using tissue containing glycogen,[142] or, if the tissue is deficient in this carbohydrate, the supplementary addition of a reducing sugar. The colloidal juices, and, to a lesser extent the soluble extractives, seem to be the growth-protecting agents.[13,38] It is perhaps unnecessary to review the catalase hypothesis,[125] according to which anaerobes were killed by hydrogen peroxide produced by dissolved oxygen, because they lacked the catalase necessary for peroxide destruction; tissues supplied the enzyme and lethal concentrations of peroxide could not be attained. This promising young hypothesis flourished until Sherman[178] found obligate anaerobes among the propionic acid bacteria which did produce catalase but showed no immunity to oxygen poisoning.

Mere chemical removal of oxygen by tissues or aerobes has been suggested;[216] in fact various reducing chemicals, such as ferrous salts, sulfides, aldehydes, formates, pyrogallol, etc., can effectively replace the tissue[64,83,86,135,201] in promoting growth. Then effective substances were found[95,105,154] which either had only slight reducing properties (e. g. hydroxylamine, phenol, etc.) or were effective in such high dilutions that no marked binding of dissolved oxygen could be attributed to them; e. g. cystine in 1:100,000 dilution. In a study of this question, Tanner and Neave[191] found hydroquinone to be even more effective than cystine, and advanced evidence to show that the effect of such substances is not the mere production of a reducing potential, but is the catalytic protection against oxidation of some step in anaerobic metabolism. These "anti-oxygenic"[134] substances are found among the metabolic products of cultures (cystine, phenols, etc.) and of living tissues (glutathion, phenols, etc.). Thus Burri and Kürsteiner's[26] view, that a culture will survive if the first generation can do so, now finds an explanation in the ability of each surviving cell to generate its own anti-oxygenic defense. Its chance for survival against a given oxygen

supply would be proportional to the food concentration, and, as Bachmann[10] has shown, dilution by inert agar increased the mortality. Heavy inoculations[106] not only give greater statistical chances for survival, but also mechanically supply the anti-catalyst among the metabolic products of the parent culture. Any means for decreasing oxygen penetration into the medium (semi-fluid cultures, glass rods or beads, coke, cotton, etc.), or any settleable matter which may carry down the cells into a concentrated colony at the bottom of the culture vessel, gives the first generation a better chance for survival. Accordingly, some of the conclusions reached in the study were that:

(a). There is no single lethal concentration of oxygen for a given anaerobe; a given concentration may or may not be lethal depending upon the age of the cell, its past history, and its present environment.

(b). Reducing potentials are the result rather than the cause of anaerobic growth.

(c). Reducing chemicals favor anaerobic development by protecting some intra-cellular components from oxidation, rather than merely by removing the free oxygen; that is they are anti-oxygenic for some step in metabolism as yet unknown.

In illustration of the last conclusion, it may be mentioned that a given number of *Clostridium sporogenes* cells suspended in normal saline (free from oxygen) were killed in one hour under certain aeration conditions, but suffered only a two percent reduction in the number of living cells under the same conditions when 0.1 per cent of hydroquinone was present.

The evidence is, then, that obligate anaerobes not only can, but must, depend upon oxygenated compounds for their supply of this element. Consequently, transformations in combined oxygen are the clues to anaerobic respiration processes. Among the facultative anaerobes there is no evidence that the initial transformations undergone by a food material on entering the cell are governed by free oxygen;[117] only the later steps in the breakdown seem to be so governed. In animal biochemistry, also, recent progress has emphasized the rôle of organic molecules as hydrogen donors and acceptors, and relegated free oxygen to the position of sweeping up the débris, thus permitting the biologically reversible processes to run to completion. Whether the organic food molecule has its energy content enhanced by the protoplasm as Mathews[122] suggests, or is activated by enzymes in accordance with Quastel's[153] view, the result is a degradation resulting aerobically in relatively simple end-products and enaerobically in more complex fragments of the original molecules. This difference in end-products leads to markedly different energy manifestations in the two types of respiration. For example, ethyl alcohol may be oxidized anaerobically (by dehydrogenation) to acetaldehyde, and this by a similar process to acetic acid, which may even be decarboxylated yielding methane and carbon dioxide; based on the heats of formation of the reactants, the following quantities of energy are made available by each step:

Alcohol to aldehyde.....11.7 Calories
Alcohol to acetic acid.....23.8 Calories
Alcohol to $CO_2 + CH_4$.....43.5 Calories

But aerobic combustion of this molecule to water and carbon dioxide would yield 328.6 Calories. This difference in energy is reflected in the greater amount of food which must be utilized anaerobically to maintain a culture; thus according to Lesser,[117] Pasteur's data indicate the need of 8 to 9 grams of sugar to give one gram of yeast aerobically, but 80 to 90 grams of sugar are needed under anaerobic conditions.

From the above characterization of strict anaerobes, it is clear that free oxygen does not play a part in their respiratory oxidations, using this word, of course, with its modern connotations to include dehydrogenation, electron transfer, etc. Much literature has accumulated in recent years on the subject of biological oxidations.

During the years 1858 to 1883, Moritz Traube[200] advocated a peroxide theory, with water as an essential medium and hydrogen peroxide formed from it as the active agent; that is, oxygen oxidized only a hydrogen of the water molecule and all subsequent oxidation was effected by the liberated OH. However, later work by Engler and Wild[60] and by Bach[4] showed that other peroxides could also be formed; thus substance A reacting with oxygen produced AO_2, a peroxide or moloxide, which in turn could oxidize more A or a second substance B:

$$A + AO_2 = 2AO \text{ or } B + AO_2 = BO + AO.$$

Meantime Hoppe-Seyler[93] and Baumann[14] advanced the idea that molecular oxygen was resolved by nascent hydrogen or other reducing agent into an atom of active oxygen and a molecule of water, the type equation being $MH_2 + O\text{-}O + H_2O = M + H_2O + H_2O_2$. This idea arose from anaerobic studies in which hydrogen evolution often occurred, and Hoppe-Seyler showed that hydrogen absorbed on palladium in water could effect many oxidations in the presence of oxygen, such as the production of phenol from benzine, of benzoic acid from toluene, etc. Similar views, postulating the resolution of the oxygen molecule into two active atoms, were reached also by Clausius[43] and by Van't Hoff.[206]

Engler and Weissburg[59] distinguished two classes of oxidations by peroxide formation; the simple case of a substance not only inducing oxidation but also itself being oxidized; for example, benzoyl-hydrogen peroxide reacting with benzaldehyde,

$$C_6H_5CHO + O_2 = C_6H_5CO.O.OH, \text{ and}$$
$$C_6H_5CO.O.OH + C_6H_5CHO = 2C_6H_5COOH;$$

and the case of a third substance, not ordinarily oxidized, but in which oxidation is produced by the peroxide of an autoxidizable substance; for example, indigo oxidation by benzoyl-hydrogen peroxide,

$$C_6H_5CO.OH + \text{indigo} = C_6H_5COOH + \text{indigo oxidation products.}$$

Many peroxide-yielding substances are now recognized, including metals like iron and zinc, non-metals like hydrogen and phosphorous, and various organic molecules such as hydrocarbons, terpenes, alcohols,

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aldehydes, acids, carbohydrates, ethers, phenols, aromatic bases and alkaloids.

A more generalized mechanism of oxygen activation has been supported by Warburg.[211] Warburg's idea is that oxygen activation is essential to the oxidation process, and that this activation is accomplished by combination with a heavy metal, usually iron (manganese or vanadium in a few marine forms). Special iron-organic combinations are found to be necessary by *in vitro* experimentation; iron-nitrogen catalysts permit oxidation only of amino acids; sugars require an iron-fructose-phosphate, and fatty acids an iron-sulfhydryl compound. Respiratory inhibition by cyanides is thus attributable to inactive iron cyanide formation.

Buchanan[25] has criticized Warburg's theory as inadequate, because it is based on so few cases of actual cell respiration; furthermore, cyanides do not wholly stop respiration, but destroy only 80 to 90 per cent of it, and some further mechanism must be assumed to explain this residual oxidation.

However, certain well established examples of metal oxide catalysis and the production by Röhrmann and Schmamine[163] of a protein mixture containing ferrous salts and hydrogen peroxide which showed the usual reactions of peroxidases, indicate the applicability of Warburg's mechanism at least in some cases.

In 1912 Wieland[213] advanced the mechanism of dehydrogenation, which is still actively competing for first place with Warburg's theory. The basis of Wieland's theory is activation and transport of hydrogen by enzymes; thus oxidations can be accomplished in the entire absence of free oxygen by union with a molecule of water followed by the removal of hydrogen by an appropriate hydrogen acceptor. These hydrogen acceptors are substances capable of undergoing reduction with moderate ease; e. g. certain dyestuffs (notably methylene blue), quinone, disulfide compounds, or even molecular oxygen. Thus, with spongy palladium and quinone or methylene blue, oxidations of the following types can be effected anaerobically:

$$RCHO + H_2O = [RCH(OH)_2] = RCOOH + H_2$$
$$CH_3CH(OH)COOH = CH_3COCOOH + H_2$$

Bach[5] has attacked this theory because Wieland assumes the addition of water by the oxidizing substance, and because his and Traube's mechanisms both fail for the oxidation of nascent hydrogen. Furthermore, his anaerobic oxidation requires enzymes not shown to exist, and the oxidizing and reducing ferments are both classed as hydrogen activators.

However, with regard to the addition of water, some reactions are very difficult to explain without such an assumption; for example, the enzymic oxidation of oxalic acid by plant seeds as reported by Thunberg:[199]

$$K_2C_2O_4 + 2H_2O = [C(OH)_2OK]_2 = 2KHCO_3 + 2H.$$

As to the requirement of reducing enzymes in tissues, Dakin[48] has expressed the opinion that "at present there appears to be little ground

for believing in special reducing enzymes in the animal body and such reductions as are observed appear to be due to coupled reactions in which the necessary energy is supplied by simultaneous oxidation."

Szent-Gyorgyi[190] and Kluyver and Donker[108] believe that neither Warburg's nor Wieland's theory alone can explain the metabolism of respiration, but that a combination of the two gives the true picture. The latter workers base the catalytic action of protoplasm on its affinity for hydrogen or oxygen; where the hydrogen affinity is strongly developed, activation of hydrogen in the respiratory substance results in its transfer to a hydrogen acceptor, but not in the evolution of the gas itself. If oxygen be the hydrogen acceptor, it must first be activated by an iron catalyst. Evolution of free hydrogen occurs only when plasma having a marked affinity for oxygen (and consequently very little for hydrogen) acts on a hydroxyl group; the two activated hydrogen atoms in this case immediately unite to a hydrogen molecule. Thus the respiration of carbohydrates and polyatomic alcohols usually leads to hydrogen evolution.

Hopkins,[92] reviewing the question, concludes that activation of the molecule must precede all oxidations, and only in some cases does oxygen activation play a part. He is of the opinion that the activation is not produced by mere capillary or surface forces, but is due to more specific forces, but is due to more specific or individualized forces, presumably enzymic in nature.

Nevertheless, selective permeability alone has been shown by Giard and Platard[72] to induce curious reactions. To illustrate, the following reactants were separated by a parchment membrane, and produced the reactions indicated:

$CuCl_2$ / Na_2S = Cu / Na_2S_4
Na_2S / fumaric acid = Na_2S_4 / H_2S + S + succinic acid
$Fe_2(SO_4)_3$ / alkaline Na fumarate = $FeSO_4$ / Na tartarate,
or even $FeSO_4$ / Na formate = Na_2CO_3.

These reactions are discussed in terms of electron transfer, a mechanism similar to that described by Clark[41] for reversible oxidation-reduction systems. Electrolytic oxidation of oxygen-free compounds in non-aqueous solvents (SO_2, $POCl_3$, NH_3) has enabled Cady and Taft[37] to demonstrate the possibility of electron transfer without the intervention of hydrogen or oxygen; the anodic processes in these studies resembled those in aqueous solutions, but the cathodic ones involved only reduction of solvent ions. Perrin[147] is of the opinion that electron transfers are preceded by the formation of addition complexes.

The precise definition of oxidation-reduction systems by Clark[42] and his associates has not yet markedly aided the formulation of respiratory mechanisms. The method still requires wider applications to simple systems and then extension to complex metabolic systems. However, Clark's method of treatment is applicable only to relatively mobile equilibria; it is not possible to determine a characteristic oxidizing or reducing potential for a single substance such as quinone, but only for for the system quinone-hydroquinone *when equilibrium has been established.* In digesting sludge the measurement of the potential is feasible,

but its interpretation is not. If, in the living cell, the oxidation of a fatty acid is governed by transformations in a sulfhydryl compound such as glutathion (as Warburg finds *in vitro*), and if these transformations alone govern the resultant reducing potential, and if this reducing potential is communicated to the medium as a whole and not merely to the cell, then fatty acid respiration could be followed by reducing potential determinations; but this causal chain has not been established, and the reducing potential of a sludge is probably determined more by the inorganic equilibria (iron, H_2S, etc.) than by the organic systems. If the hydrogen found in sludge digestion gases (about two per cent by volume) were arising from some mobile equilibrium, a reducing potential of about -0.35 volt (referred to the normal hydrogen electrode) would be indicated at a pH value of 7.0. This is much more negative (more highly reducing) than reported values[52] for the cysteine or glutathion systems. However, the only evidence that hydrogen evolution is the result of a reversible process, is the fact that digesting sludge will absorb large volumes of gaseous hydrogen; whether the absorption is effected by the same mechanism as the evolution is unknown. The data are, at present then, entirely inadequate for a profitable discussion of this topic.

A consideration of free energies, even if these were known for all the metabolic participants, does not determine the mobility of an equilibrium; as Conant[44] states "there is, in general, no necessary relationship between the speed and the free energy change of a reaction." He mentions racemization as an illustration,—a process showing zero energy change from one pure antipode to the other, but with widely varying reaction rates for different substances. The oxidation or reduction potential of the active agent becomes important only if the reaction rate is controlled by a reversible equilibrium involving this agent; then a "powerful" reducing agent will produce a higher reaction velocity than a "mild" one. Conant is quantitatively defining the "strong" and "weak" oxidizing agents for the irreversible transformations to which Clark's method is inapplicable. This will be a valuable contribution, since most biochemical degradations are irreversible reactions.

Little help can be obtained, then, from reduction potentials and free energies in formulating detailed mechanisms, and recourse must be had to speculation followed by experimental verification. But whatever may be the exact course of a biochemical transformation, it requires the catalytic action of a cell. One or more of the participating molecules must undergo a modification or activation. As previously mentioned, Warburg stresses the activation of oxygen and Wieland the activation of hydrogen. Mathews[122] suggests that the whole molecule undergoes activation in the presence of protoplasm and regards the only difference between molecules of living and of dead matter as an enhanced energy content of the former. A vague picture of this activation is presented in terms of electronic orbits.

A more useful concept of activation has been advanced by Quastel;[153] this concept postulates the existence of activating enzymes as

discrete points externally located on the bacterial integument (this idea has been reached also by Waldschmidt-Leitz[209] of the Willstätter school), and the adsorption of the food molecule at these enzymic foci prior to activation. The configuration assumed by the activated molecule is governed by J. J. Thompson's rule[195] of electron shift under the influence of a polarizing field, since the enzymes are regarded as regions of high electrostatic intensity. The subsequent fate of the activated molecule is conditioned by the nature of the other substances present in the medium. The simplest case is that of a double-bonded carbon pair, such as $C_2 = C_1$, or $\dot{C}::\dot{C}$.; under the influence of the polarizing field applied at C_1 the electrons enter new orbits, and C_1 may become saturated (relative to C_2) by acquiring protons from C_2, giving the activated structure $—\overset{1}{\underset{1}{C}} — CH_2 —$ and leaving the distal carbon atom very reactive. If a radicle, R, is introduced into the chain to give the compound —CH = C-R, its effect on the adjacent carbon atom will depend upon the electronic nature of the radicle; if the radicle requires an electron to complete its octet, the alpha-carbon will become more positive and will repel other entering groups of a positive nature. Thus, the OH^- radicle requires an electron to complete its octet $(:\ddot{\underset{\cdot}{O}}:H)$; consequently when introduced into the carbon chain, the electrical nature of the adjacent carbon atom is modified,—Quastel simply says it becomes more positive; Lewis[118] would say the bonding pair of electrons is drawn away from the carbon atom, thus $\begin{matrix} H & \\ :C & :\ddot{O}:H \\ H & \end{matrix}$. The result is that when such a chain is activated by enzymes, this adjacent carbon atom repels protons (hydrogen ions) or attracts negative groups. The reverse effect is shown by introducing a radicle which contains one electron more than is required by the octet. Activated molecules of the following types are thus produced:

$$—CH = CH — COOH \text{ becomes } —\overset{V}{C} — CH_2COOH$$

$$—CH = CH — CH_3 \text{ becomes } —CH_2 — \overset{V}{C}CH_3$$

$$—CH = CH — CH_2COOH \text{ becomes } —CH_2 — \overset{V}{C}CH_2COOH$$

The same considerations have been applied to saturated molecules of various types in order to predict what change the compound will undergo in the presence of bacterial enzymes. By way of illustration, we may consider the probable behavior of propionic acid in the presence of a hydrogen acceptor (methylene blue) and living cells (e. g. *Bact. coli*). The molecule on activation assumes the structure $CH_3\overset{V}{C}COOH + 2H^+$ and, if the hydrogen is removed, could become acrylic acid;

but (in Quastel's terminology) the alpha carbon attracts negative electricity on account of the proximal CH_3 and positive electricity due to the carboxyl group; therefore, it is more apt to add HOH and produce lactic acid, and this latter is what experiment shows.

This mechanism was developed by Quastel to explain the results of his extensive studies on the transformations of organic molecules in the presence of hydrogen acceptors and washed cells of *Bacterium coli.* Of 103 substances examined, 56 were activated by *B. coli,* and it is unlikely that these cells contain 56 specific hydrogen-activating enzymes or hydrogen "transportases." Quastel's picture requires far less enzymic specificity, and in addition explains the high reducing power of succinic as compared to glutaric acid, of alanine compared to glycine, and similar observed results.

With regard to the hydrogen acceptors necessary to permit transformations in the activated molecules, Quastel recognizes three kinds:

1. Those activated or enhanced by the cell, such as nitrates, chlorates and fumarates.

2. Those independent of the cell, such as dyes and palladium.

3. Oxygen, perhaps inactivated, or if not, then activated by iron, by sulfhydryl compounds or by peroxide formation with groups such as —CH = CH—, —CH = O and —CH = N—.

Raper[159] is of the opinion that the anaerobic oxidases are the enzymes which activate the hydrogen of the substrate; they thus convert substances which are not commonly regarded as reducing agents into such, and permit the oxidation even of stable fatty acids.

From this brief literature review, the most useful picture of biological oxidation seems to be the following. The molecule to be oxidized is activated, probably by anaerobic oxidases, whereby its hydrogen is rendered labile; the hydrogen may be discharged as a gas by certain types of protoplasm (Kluyver and Donker) as in many anaerobic carbohydrate fermentations, or it may be taken up by some other molecule acting as a hydrogen acceptor. After the disposal of this hydrogen, the parent molecule may react in various ways depending upon its chemical structure and for many molecules Quastel's mechanism predicts the final products. In aerobic oxidation, the hydrogen acceptor is either molecular or activated oxygen. The anaerobic oxidative mechanisms seem to fall into five groups:

(1) Oxidation of carbon atoms by intramolecular transformations:

$C_6H_{12}O_6 = 3CH_3COOH$
$C_6H_{12}O_6 = 2CH_3CHOHCOOH$
$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$
$C_6H_{12}O_6 = 3CH_4 + 3CO_2$

(2) Oxidation by dehydrogenation and discharge of molecular hydrogen:

$C_6H_{12}O_6 = 2CH_3COCOOH + 2H_2$
$C_6H_{12}O_6 = CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$

(3) Oxidation by dehydrogenation and acceptance of the hydrogen; many of the *in vitro* experiments of Wieland are of this type; e. g.

CH_3CH_2OH + methylene blue = CH_3CHO + leuco-methylene blue.

(4) Oxidation by water with the discharge of molecular hydrogen:

$C_6H_{12}O_6 + 6H_2O = 6HCOOH + 6H_2$

$C_6H_{12}O_6 + 6H_2O = 6CO_2 + 12H_2$

(5) Oxidation by water with acceptance of the hydrogen, usually by other carbon atoms in the same molecule:

$2\,C_6H_{12}O_6 + 3H_2O = 3C_2H_5OH + 6HCOOH.$

Frequently two or more of these mechanisms may operate either simultaneously or in response to changing environmental conditions within the culture. This variability of mechanism is shown especially by those organisms whose protoplasm has a hydrogen-activating intensity sufficient to produce a hydrogen concentration equal to its overvoltage and permit the discharge of this gas.

Organisms which do not reach the reducing potential needed for hydrogen discharge have to transfer the hydrogen to other atoms or molecules, and the metabolic products of such cultures will contain reduced products like H_2S, CH_4, alcohols, aldehydes, etc.

Mechanism of Fatty Acid Degradation. Acids above acetic in the homologous series produce more carbon dioxide than could be contributed by the carboxyl group of the acid; thus for propionic acid, 74 grams (1 mole) of the acid could yield 44 grams of CO_2, or 130 grams could yield 77.3 grams of CO_2; but reference to the tank run on propionic acid shows that 130 grams yielded 96.3 grams of CO_2. The 19 grams excess CO_2 represents 13.8 grams of oxygen which came from somewhere other than the metabolized acid; neither the oxygenated inorganic salts present nor the original inoculum showed any such change in weight, so the oxygen must have come from water. Also since practically no gaseous hydrogen is discharged, the respiratory mechanism belongs to the fifth class listed above.

Bearing in mind that this bacterial degradation is to obtain energy, the maximum energy yield would result from complete oxidation of the molecule:

$2C_3H_6O_2 + 7O_2 = 6CO_2 + 6H_2O + 365$ Calories.

But with water as the only oxidizing agent, no such complete combustion can be effected unless the hydrogen of the water molecule is discharged as gas. The organisms responsible for these fatty acid degradations have not the ability to discharge hydrogen gas (not even from carbohydrates), so some of the carbon atoms have to be sacrificed as hydrogen acceptors and "wasted" as methane. The result is a decomposition represented by the equation:

$4C_3H_6O_2 + 2H_2O = 5CO_2 + 7CH_4 + 20$ Calories

A word of explanation is required regarding the energy change represented by this equation, because if heats of formation are merely

substituted into the equation as it stands, the reaction is endothermic. However, this reaction does not represent the system on which the microorganisms operate, and from which they derive their energy: the bacteria are concerned only with the transformation of a dilute aqueous solution of the acid into H_2CO_3 and dissolved methane; the subsequent dissolution of methane and decomposition of H_2CO_3 are extracellular reactions for which heat energy has to be supplied from the surroundings. For the cell then, the reaction is:

$$4\ C_3H_6O_2\ (\text{dil. soln.}) + 7\ H_2O\ (\text{liquid}) = 5\ H_2CO_3\ (\text{dil. soln.}) + 7\ CH_4\ (\text{dil. soln.})$$

$$\underbrace{(4 \times 123) + (7 \times 68.38)}_{971\ \text{kg-calories}} = \underbrace{(5 \times 167.5) + (7 \times 22)}_{991.5\ \text{kg-calories}}$$

The heats of formation are taken from the International Critical Tables, Vol. V, pp. 162, 169 (1929) with two changes: no heat of solution is given for propionic acid for the dilution employed, but the magnitude of this correction* is within the uncertainty in the value

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*Note: This heat of solution is —0.3 kg. cal. per mol for moderately concentrated solutions, and + 0.6 kg-cal. for infinite dilution; the heat of formation of the acid is uncertain by 1.0 kg-cal.

of the heat of formation assigned to propionic acid, so the uncorrected heat of formation has been used; also no heat of solution is given for methane, but since the tables give CO = 3.4, C_2H_2 = 4.1, O_2 = 3.3 and H_2 = 1.4, we have assumed a value of 3 for CH_4 and added this to the tabular value for the heat of formation of gaseous CH_4 (viz. 19).

Similar calculations on the other fatty acids show the following values:

Acid	Available kg-calories per mol acid
Formic	56
Acetic	3
Propionic	20
n-Butyric	8
n-Valeric	29
Stearic	3
Lactic	39

The reactions on which these calculations are based are the series of water oxidations shown in Table XXI. The observed CO_2:CH_4 gas ratios in the table show how closely the degradations follow the simple water-oxidation equation. The agreement for butyric and valeric acid is poor because experimental ratios have only been determined on bottle tests and not yet by tank runs.

TABLE XXI

FATTY ACID DEGRADATION REACTIONS

	Fatty acid	Reaction	Observed CO_2:CH_4
Formic	$4CH_2O_2 - (2H_2O)$	$= 3CO_2 + CH_4$	.....
Acetic	$C_2H_4O_2$	$= CO_2 + CH_4$	1.03:1
Propionic	$4C_3H_6O_2 + 2H_2O$	$= 5CO_2 + 7CH_4$	5.04:7
n-Butyric	$2C_4H_8O_2 + 2H_2O$	$= 3CO_2 + 5CH_4$	2.7 :5
n-Valeric	$4C_5H_{10}O_2 + 6H_2O$	$= 7CO_2 + 13\ CH_4$	6.7 :13
Stearic	$C_{18}H_{36}O_2 + 8H_2O$	$= 5CO_2 + 13\ CH_4$	.....
Lactic	$2C_3H_6O_3$	$= 3CO_2 + 3CH_4$	1.06:1

This mechanism in which some carbon atoms accept hydrogen to give methane explains all the gas ratios found by experiment, and the number of molecule of water required by one molecule of fatty acid is found to increase in a regular manner while ascending the homologous series. This number is equal to $\frac{n-2}{2}$, where n = the number of carbon atoms in the fatty acid. The general equation for the anaerobic oxidation of the saturated monocarboxylic acids is, accordingly:

$$C_n H_{2n} O_2 + \frac{(n-2)}{2} H_2O = \frac{(n+2)}{4} CO_2 + \frac{(3n-2)}{4} CH_4$$

While ascending the homologous series above acetic, therefore, the weight of gaseous products exceeds the weight of acid digested by an increasing percentage (Figure 4), attaining a purely hypothetical maximum of 164 per cent of the acid decomposed for an infinite number of carbon atoms. The percentage of methane in the gaseous products also increases to a hypothetical limit of 75 per cent by volume, though due to the solubility and chemical retention of carbon dioxide in the medium, the gases actually collected often contain 80 to 85 per cent of methane.

The complex values of the CO_2:CH_4 ratios, instead of simple 1:1 ratios indicating alternate oxidation and reduction occur only because water as the oxidizing agent is burdened with two superfluous hydrogen atoms. The latter can be eliminated only by dehydrogenation, either of the carbon chain or of the water molecule. It follows that if oxygen were supplied (anaerobically) without the accompanying hydrogen, the simple 1:1 ratio should be obtained even from an acid like propionic. This oxygen addition can be accomplished by replacing one hydrogen in the acid by a hydroxyl group; this does not change the total number of hydrogen atoms, but is equivalent to half oxidizing the carbon atom. Therefore, while propionic acid gives a 5:7 ratio, the alpha hydroxypropionic (lactic) acid should give a 1:1. The value found by experi-

ment was 1.06:1 for the CO_2:CH_4 ratio. Graphically the difference between propionic and lactic may be illustrated thus:

CH_3CH_2COOH	$CH_3CHCOOH$
OH_2	OH
OH_2	OH
CH_3CH_2COOH	$CH_3CHCOOH$

In the latter case (lactic) all of the elements to form $3CH_4$ and $3CO_2$ are present in the two molecules of acid; the intervention of an external oxidizing or reducing agent is not required. But in the former case

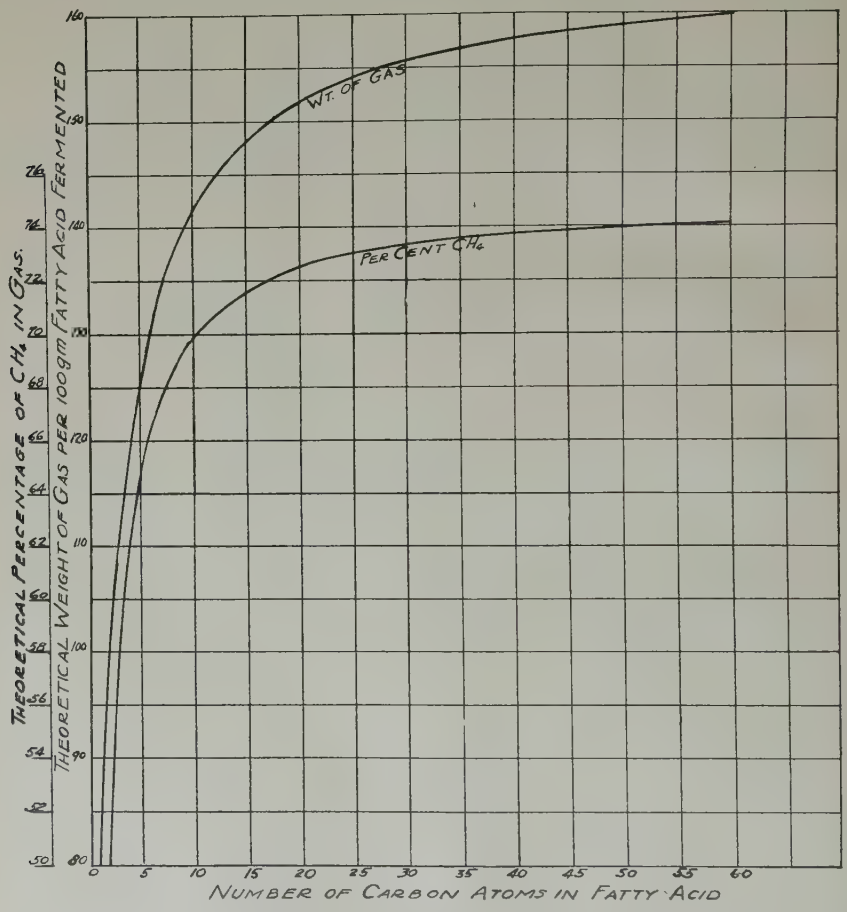

Fig. 4—Theoretical weight and methane content of fermentation gases.

(propionic), in order that one alpha-carbon may be oxidized by two molecules of water, two molecules of hydrogen must be disposed of; in these cultures there is no mechanism for discharging gaseous hydrogen, hence it must be removed by hydrogen acceptors. But the only

hydrogen acceptor present is propionic acid, or rather the alpha-carbon atom of this acid, and disposal of the hydrogen consequently results in the reduction of two additional propionic molecules:

$$\begin{array}{l} CH_3CH_2COOH \\ \quad H_2 \qquad\qquad \longrightarrow 4CH_4 + 2CO_2 \\ \quad H_2 \\ CH_3CH_2COOH \end{array}$$

The initial oxidation step yields $3CH_4 + 3CO_2$, and the disposal of hydrogen yields an additional $4CH_4 + 2CO_2$, the over-all ratio being $7CH_4 + 5CO_2$, which is the ratio found by experiment. This illustrates the above statements that odd-numbered gas ratios are attributable to the interfering hydrogen of the water molecule, and that the production of methane in this respiratory process is largely an ancillary result of hydrogen acceptance. In fact this type of hydrogen disposal is common to organisms which lack the mechanism for discharging molecular hydrogen, in contrast to others (*B. coli* and anaerobic clostridia) which do produce hydrogen. The former group would ferment even glucose and glycerol to methane (as experiment shows they do), while the latter group would discharge hydrogen instead of producing methane.

Another line of confirmatory experimentation suggests itself, namely, the addition of hydrogen acceptors along with the propionic acid to take care of the hydrogen of the water molecule and permit the propionic acid to give a 1:1 gas ratio. Preliminary experiments in this direction have not been successful. Succinic acid was the first acceptor tried, because if it breaks down by taking up a hydrogen molecule, it can give a 1:1 gas ratio; this ratio is desirable because any other might obscure the results of the propionic degradation. The succinic reaction is:

$$COOHCH_2CH_2COOH + H_2 \longrightarrow 2CO_2 + 2CH_4.$$

The addition of 2.36 grams of succinic acid and 1.48 grams of calcium propionate to a Söhngen sludge actually gave a CO_2:CH_4 ratio of 1:0.73 to 1:0.79, but unfortunately control tests on the succinic alone showed that it could break down according to the equation:

$$4C_4H_6O_4 + 2H_2O \longrightarrow 9CO_2 + 7CH_4$$

In addition, the gas analyses showed that the succinic acid had incrased the evolved CO_2 by 1.82 grams; most of the succinic had thus oxidized. In other words, the propionic had acted as the hydrogen acceptor and permitted the oxidation of succinic acid.

Glycocoll was similarly tried as a hydrogen acceptor, since it could be reduced to acetic acid:

$$CH_2(NH_2)COOH + H_2 \longrightarrow CH_3COOH + NH_3;$$

and the acetic could ferment to a 1:1 ratio without obscuring the results. However, this amino acid practically stopped gas production until it had nearly all been metabolized (as shown by decrease in free amino nitrogen), and a gradual drifting of the CO_2:CH_4 ratio was observed from 1:2.51 early in the experiment to 1:0.94 toward the end. Here again, interpretation of the findings is difficult.

The steps in the transfer of oxygen from the water molecule to the CH_2-group of the acid cannot yet be stated. Beta oxidation, as ordinarily pictured, is not the mechanism for the lower fatty acids, because it would involve the evolution of appreciable amounts of hydrogen. In the case of the higher fatty acids in a complex sludge mixture, beta oxidation was stated to be a possibility because hydrogen acceptors, or the hydrogen absorbing property of sludge might take care of the hydrogen; but in these cultures no hydrogen acceptors (other than the fatty acid molecules) are present, and the inoculating sludge cannot be absorbing this hydrogen, because Söhngen sludges have been shown not to have this property. The only possibility is hydroxylation, with other molecules of the acid acting as hydrogen acceptors; thus for propionic:

$$2CH_3CH_2COOH + 2H_2O \longrightarrow 2CH_3CH(OH)COOH + 2H_2$$
$$2CH_3CH(OH)COOH \longrightarrow 3CH_4 + 3CO_2$$
and $$2CH_3CH_2COOH + 2H_2 \longrightarrow 4CH_4 + 2CO_2$$

The total gas, $7CH_4:5CO_2$, corresponds to the experimental values.

For butyric acid it will be necessary to provide a mechanism not only for hydroxylation of a carbon atom, but also for the resultant hydroxy acid to recombine with the hydrogen removed, thus:

$$2CH_3CH_2CH_2COOH + 2H_2O \longrightarrow 2CH_3CH(OH)\ CH_2COOH \longrightarrow + 2H_2$$
$$2CH_3CH(OH)CH_2COOH + 2H_2 \longrightarrow 3CO_2 + 5CH_4.$$

The degradations not only involve oxidation by water but also an intramolecular oxidation-reduction process.

In conclusion, a word or two must be said about the excess CO_2 and the hydrogen in the gases from fatty acid metabolism. Since no other products, indicating side reactions, have been found, the gases in question have been attributed to the internal metabolism of the cell. That is, the respiratory degradation, which is shown by experiment to convert 95 per cent or more of the acid into CO_2 and CH_4, supplies the energy required for movement, growth and anabolism. When only a simple fatty acid is fed, a part of the acid must undergo profound changes in structure in the anabolic process if it is to become a part of the cell protoplasm. The hydrogen and excess CO_2, then, are suspected of arising during this anabolism. Experiments are still in progress, with three types of reactions in mind:

(1) $CH_3COOH + 2H_2O = 2CO_2 + 4H_2$
(2) $RCH_2COOH + NH_3 = RCH(NH_2)COOH + H_2$
(3) the production of higher homologues (e. g. succinic from acetic).

The experimental evidence favors some form of oxidative cycle, such as amino acid production followed by hydrolysis to the hydroxy acid, and perhaps oxidative degradation of this by water. However, a profitable discussion of this topic must await further experimental results.

Summary

The following aspects of the degradation of carbonaceous matter have been discussed:

a. A rough separation of the carbonaceous constituents of sewage sludge and fermentation tests on these and related compounds have shown grease (soaps and fats) to be an important producer of methane.

b. Sewage grease, in this locality, has been shown to consist of about equal parts of lime soaps and triglycerides.

c. Digestion of grease has been shown to occur in actively digesting sewage solids and in certain types of carbohydrate and protein cultures. The digestion products are lower fatty acids.

d. Digestion studies on lower fatty acids have proved them to be important precursors of methane, whether they arise in protein, fat or carbohydrate degradation.

e. A brief review of the biological oxidation theories is given.

f. The methane degradation of fatty acids is shown to be an oxidation caused by water, and a definite relation has been established between the length of the carbon chain and the number of water molecules utilized in its decomposition.

V

GENERAL CONCLUSIONS

1. The anaerobic degradation of some of the dominant constituents of sewage sludge has been studied with reference to the fate of these substances in the sludge digestion process in sewage treatment.

2. The major nitrogenous constituents were shown to be urea, nucleoproteins and simple proteins, and their degradation products. The hydrolysis of urea to ammonium carbonate was rapid and complete. Insoluble proteins were hydrolyzed and peptised slowly in acid sludges (pH 5.0) but rapidly above pH 6.4. Fatty acids, ammonia and free amino acids were the normal products, but amines also were produced in acid sludges. Fatty acids were decomposed with the production of methane and carbon dioxide. Amino acids were in part re-synthesized into bacterial protein. The purine fraction of the nucleoproteins suffered almost complete destruction. During the remobilization in forms of nitrogen, not more than five per cent of that present escaped as gaseous nitrogen. Ammonia, living bacteria and amino acids were the main nitrogenous end-products.

3. Besides proteins, the sewage solids were shown to contain grease (soaps and fats), crude plant fiber and humus-like bodies. Grease degradation was demonstrated, and lower fatty acids, methane and carbon dioxide established as end-products; thus grease was proved to be an important source of methane in digestion tank gases. The degradation of lower fatty acids was studied to establish the biochemical mechanism involved.

4. For the degradation of lower fatty acids (and by inference for higher ones) an anaerobic oxidative mechanism has been demonstrated in which water acts as the oxidizing agent. Methane and carbon dioxide were produced in nearly theoretical yields. This mechanism probably has the wide application in anaerobic processes that the beta-oxidation mechanism has in aerobic metabolism.

VI

ANALYTICAL METHODS

In addition to the Standard Methods of the American Public Health Association, the following determinations have been made and accordingly require a few comments.

Proteins. The usual trichloracetic acid method[90] was used for protein precipitation in spite of the partial hydrolysis which it is said to effect,[47] because mercuric sulfate and tungstic acid precipitants absorb too much of the free amino acid. Total nitrogen in the precipitate was determined by Kjeldahl digestion, except for minute amounts of protein, when the colorimetric method[121] based on the xanthoproteic reaction is preferable. In the trichloracetic filtrates, diacipiperazines can be separated from amino acids by barium hydroxide and carbon dioxide treatment.[23]

The peptones can be removed in the usual way by tungstic acid[148] and the diamino acids by phosphotungstic acid.

Amino Acids. The Sörensen titration[184] method has given low results in our hands, but either the Van Slyke[87] or the Folin[66] method is satisfactory; thus with peptone and gylcocoll solutions, the following values were found (in milligrams N per cc.):

	Peptone	Glycocoll
Theoretical	.....	0.1000
Sörensen	.....	.074
Van Slyke	0.3975	.0987
Folin	0.398	.103

For the Van Slyke method, the micro-apparatus has been used, and for most samples a four-minute standing and a four-minute shaking period have been adopted for the nitrous acid reaction. Under these conditions, the interfering effect of ammonia was corrected for by the following factors:

Temperature	Per cent of the NH_3-N reacting
21	34
23	39
25	45
27	51
29	56
31	62

In general, removal of the ammonia by permutit is preferable. Urea also interferes (see below) but can be destroyed by urease. Both

of these substances should be removed if amino-purines are to be determined in the Van Slyke apparatus because these require long reaction periods (15 to 30 minutes). The Van Slyke method determines only half of the nitrogen of arginine and tryptophan, one-third that of histidine and none of the proline and oxyproline.[204] If acetic acid is used in the apparatus, amides and urethane do not react (they do with HCl), biuret gives only one nitrogen (but 2 to 3 with HCl) and creatinine only one.[150]

The Folin reagent, sodium 1:2-naphthoquinone 4-sulfonate, gives a red color with ammonia, amino acids and amines; blue with indol; and no color with urea, uric acid, creatinine and hippuric acid. Arginine however gives only about 16 per cent of its nitrogen.

The individual amino acids have been separated by phosphotungstic acid, etc., according to the common practice. For the hydrolysis of protein in sludges, however, HCl was found to destroy 20 to 30 per cent of the amino nitrogen (apparently by oxidation catalysed by $FeCl_3$) and H_2SO_4 had to be used in its stead.

Tyrosine is most conveniently determined colorimetrically[220] by Millon's reagent; arginine by arginase[97] or flavianic acid,[112] and cystine by the following modification of Okuda's iodate method:[139]

The earlier bromine method of Okuda was not specific for cystine, but his iodate method is uninfluenced by all the other amino acids. It depends upon the fact that, in acid solution, cysteine is more or less completely oxidized to cysteic acid. Whatever the exact reaction may be, if the conditions are strictly standard, an equivalent amount of KIO_3 is consumed and any excess liberates free I_2 which indicates the end-point. Okuda depends upon the yellow color of the iodine and takes as the end-point a yellow which persists for some minutes. This is not very sensitive, so we prefer to add a few drops of starch paste; this not only gives a sharper end-point but also enables one to titrate in a yellow digestate without complete clarification with bone-black. Unfortunately the reaction is an incomplete equilibrium and the end-point depends upon the cencentration of cysteine and acid, and temperature. However by adopting standard conditions and a definite blue shade as end-point, accurate results are possible.

The procedure in general is: (1) for cysteine alone,—the solution is adjusted to a definite concentration of HCl and titrated; (2) for cystine alone,—the solution is reduced with zinc dust, filtered, the HCl concentration adjusted, and the solution titrated; (3) for both cysteine and cystine,—the solution is divided into two parts; cysteine is titrated directly in the one; the other is reduced for cystine plus cysteine as cysteine, then the pre-existing cysteine is subtracted from this total to give cystine in the original.

Standard solutions required are:

N/10 NaOH or other convenient strength (for adjusting the HCl concentrations).

Exactly 4 per cent HCl (adjusted by titration against the NaOH).

Exactly 2 per cent HCl (adjusted by titration against the NaOH).

5 per cent KI (free from iodate) in distilled water.

N/300 KIO_3 (2.14 gm. in 3 li. of exactly 2 per cent HCl) standardized against pure cysteine (or cystine) at different temperatures so as to get the variation of end-point with temperature.

The titrations are carried out in the following manner. The unknown solution containing some HCl (since cystine and cysteine are unstable in alkaline solution) is reduced preferably to a volume of about 50-60 cc. If it contains cystine to be titrated, 0.3-0.5 gm. Zn dust are added and reduction allowed to proceed for 30 minutes; filter through paper and wash with a few cc. of water. This filtrate, or the original solution (if reduction was not needed), is measured,—say the vol. = 27 cc.; 1 cc. is removed, diluted with water, and titrated with N/10 NaOH to determine its HCl concentration. The remaining 26 cc. are then adjusted with 4 per cent HCl or water, as required, to give 2 per cent HCl in the solution. To this adjusted solution are then added 5 cc. 5 per cent KI, 5 cc. 4 per cent HCl, about ½ cc. starch paste and the mixture titrated to a distinct and fairly permanent blue color. The temperature of the titrated solution is noted and from the appended table the factor of the solution is obtained. For example: the 26 cc. require 0.55 cc. N/300 KIO_3 at 27°. At 27°, 1 cc. KIO_3 = 2.14 mg. cystine, therefore 2.14 x 0.55 = 1.177 mg. cystine in 26 cc., therefore in 27 cc. = 27 x 1.177/26 = 1.22 mg. cystine in sample. Since 1 cystine (mol. wt. 240) = 2 cysteine (mol. wt. 121), the factor is practically the same for both; for extreme accuracy the factors given in the table (for cystine) should be multiplied by 242/240 for cysteine.

Temperature	Mg. cystine per cc. N/300 KIO_3
18	2.37
20	2.32
22	2.27
24	2.22
26	2.17
28	2.11
30	2.06

For Sewage. 500 cc. or more are acidified with 10 cc. conc. HCl and evaporated down to somewhat less than 100 cc. (this not only concentrates the sample but also expels H_2S which would be titrated). Dilute to exactly 100 cc. and divide into two halves.

One-half is filtered, 1 cc. titrated with N/10 NaOH to get its percentage of HCl, the concentration adjusted to 2 per cent and the cysteine titrated as above.

To the other half, about 0.5 gm. Zn dust is added and the mixture filtered at the end of 30 minutes. 1 cc. is titrated and the HCl concentration adjusted, etc., as above, giving cysteine + cystine.

For Sludge Liquors. 200 cc. may be treated as for sewage.

Solid Sludges. These require hydrolysis to liberate cystine from the proteins and with the usual methods, the results are believed to be of doubtful significance. The usual method is to reflux 200 cc. of sludge with 600 to 800 cc. conc. HCl for 6-8 hours, distil off the HCl under a vacuum (15 mm. press. or less), take up the nearly dry residue with water, boil with bone-black, filter, and use the filtrate for the titrations above. It is believed that iron in the sludge ($FeCl_3$) destroys most of the cystine. No method has been worked out for removing iron. An enzymatic digestion, such as is used in the usual isolation of cystine from pure proteins, might be worked out.

As tests on the modified Okuda method, the following examples are typical:

(1) To peptone broth (containing glucose), 0.01 per cent cystine was added and the broth sterilized. Analysis then showed cysteine = O, cystine = 0.0099 per cent.

(2) Liquor from a sludge digestion bottle was analyzed, showing cysteine = 3.3 p.p.m., and cystine = 9.9 p.p.m.

(3) A strong raw sewage (fresh sample) showed cysteine = 0 and cystine = 3.3 p.p.m.

After hydrolysis as outlined for solid sludges, this sample showed less than 3.3 p.p.m. total cystine, thus indicating that a part at least of the cystine was destroyed.

(4) An old glucose peptone broth culture of *Clostridium sporogenes* was titrated showing

H_2S =	10.6 p.p.m.
cysteine =	31.6 p.p.m.
cystine =	4.2 p.p.m.

Urea. This compound is best determined by the standard urease method[87] after removing the free ammonia by aeration or permutit.

It can be determined by the Van Slyke apparatus, using a 20 to 30 minute reaction time; thus for a solution containing 0.1998 milligram of urea-N per cc., there was found:

Reaction time Minutes	Urea-N
2	0.0588
15	.1314
30	.2074

Urea may be determined more quickly in the Van Slyke apparatus by adding the sample to 1 cc. of Millon's reagent and 5 cc. of chloroform in the reaction chamber, and measuring the evolved nitrogen (the chloroform takes up the CO_2); ammonium salts, uric acid, amino acids and purines are said not to interfere.[51] We find 95 per cent of the

ammonia will react in two hours. The following is an example of the method:

	Mg. Urea-N per cc.
Van Slyke	0.1998-0.2074
Millon reagent	.2043- .2049

The widely used xanthhydrol method[126] was found to recover urea only in concentrations above 300 p.p.m.

Amines. Amines can be separated from ammonia by sodium cobaltinitrite[116] or mercuric oxide[212] and the amine determined in the Van Slyke apparatus using 15 minutes reaction time and 5 minutes shaking; this obtains only mono-amines. The dry chlorides (including NH_4Cl) may be extracted with chloroform[67] in which the di- and tri-amines are soluble (ethylamine hydrochloride is an exception, being soluble).

Purine Bases. Besides the standard metal precipitants,[39] the following are useful. Uric acid alone can be removed as zinc urate[133] and the filtrate used for the detection of other purine bodies; 40-50 cc. of sample are treated with 5 drops each of 5 per cent NaOH, 0.5 per cent p-aminophenol (or better metol-hydrochloride) and 1 per cent potassium (or sodium) persulfate; a yellow or yellowish-brown color (maximum in 15-20 minutes) is a positive test and is sensitive to 1 part in 100,000.[149] The usual Folin phosphotungstic acid method for uric acid is also positive with alloxan and alloxantin, but not with urea, ureides, guanidine, creatinine, amino acids, indol derivatives, glucose, acetone bodies, fats or lipoids.[75]

In the examination of sludges for purines, pyrimidines, picoline carboxylic acid, paraffin hydrocarbons, lignoceric acid, etc., the procedure used on soils by Shreiner and Shorey[180] has been followed.

Volatile Fatty Acids. Total volatile acid is determined on a 200 cc. sample acidified with 2 cc. concentrated H_2SO_4 and distilled until 150 cc. have come over; the distillate is titrated with 0.1 N NaOH and the alkali used calculated to acetic acid. The bottom of the distilling bulb is protected by a plate of asbestos board having a circular hole cut out for the flask; this protection prevents superheating the flask walls and consequent decomposition of chlorides and of organic matter.

The volatile acid components of a mixture have been determined by the Duclaux method[54] by distilling a known amount of total acid contained in a volume of 110 cc. and collecting ten 10-cc. fractions of distillate which are titrated separately. A comparison of the rate at which the acids come over with the rates for pure components indicates the nature of the acid mixture. Distillation rates for the pure acids, as determined on our apparatus for acids carefully purified by G. E. Symons of this laboratory, are given in Table XXIII and Figure 5.

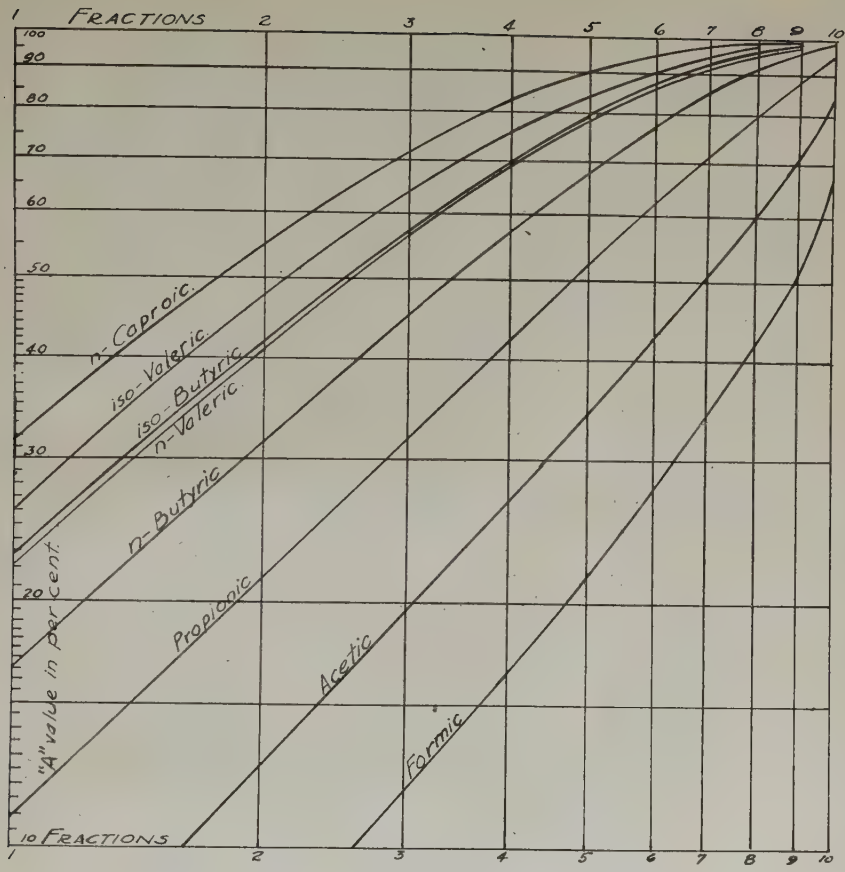

Fig. 5—Duclaux distillation curves.
For pure acids

TABLE XXII

DUCLAUX DISTILLATION CONSTANTS FOR PURE ACIDS

Fraction number	Formic		Acetic		Propionic		n-Butyric		iso-Butyric		n-Valeric		iso-Valeric		n-Caproic	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	3.5	5.3	6.2	7.3	10.8	11.4	16.6	16.7	22.6	22.8	22.1	22.4	26.0	26.2	31.4	31.5
2	7.5	11.3	12.6	15.3	21.5	22.6	31.5	31.7	41.7	42.1	40.8	41.5	47.4	47.8	55.0	55.2
3	11.7	17.6	19.7	23.6	31.9	33.6	45.1	45.4	57.3	57.9	56.5	57.4	63.7	64.2	71.8	72.1
4	16.4	24.7	26.9	32.2	42.2	44.4	57.4	57.7	69.8	70.5	69.2	70.2	76.3	76.9	83.0	83.4
5	21.6	32.4	34.5	41.3	52.1	54.8	68.2	68.5	79.7	80.5	79.1	80.4	84.8	85.5	90.2	90.6
6	27.4	41.0	42.4	50.8	61.7	64.9	77.5	77.9	87.1	88.0	86.5	87.9	90.8	91.5	94.6	95.1
7	34.1	51.1	50.9	60.9	70.8	74.5	85.3	85.7	92.4	93.3	91.9	93.4	94.6	95.4	97.2	97.7
8	41.9	62.7	60.3	72.1	79.5	83.7	91.6	92.1	95.9	96.8	95.3	96.8	97.4	98.2	98.5	99.0
9	51.9	77.7	70.9	84.8	87.6	92.2	96.1	96.6	98.1	99.0	97.5	99.0	98.3	99.1	99.1	99.6
10	66.7	100.	83.6	100.	95.0	100.	99.5	100.	99.0	100.	98.5	100.	99.2	100.	99.5	100.

NOTE:

A=the percentage of the total acid which is put into the flask that comes over by the time the indicated number of fractions is reached; e. g. for formic, by the time the seventh fraction has been collected, 34.1 per cent of the acid in the flask has come over.

B=a similar percentage, but based on the total acid coming over in 10 fractions as 100 per cent; e. g. for formic, 41.0 per cent of the total acid that will come over in 10 fractions is contained in the first six.

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